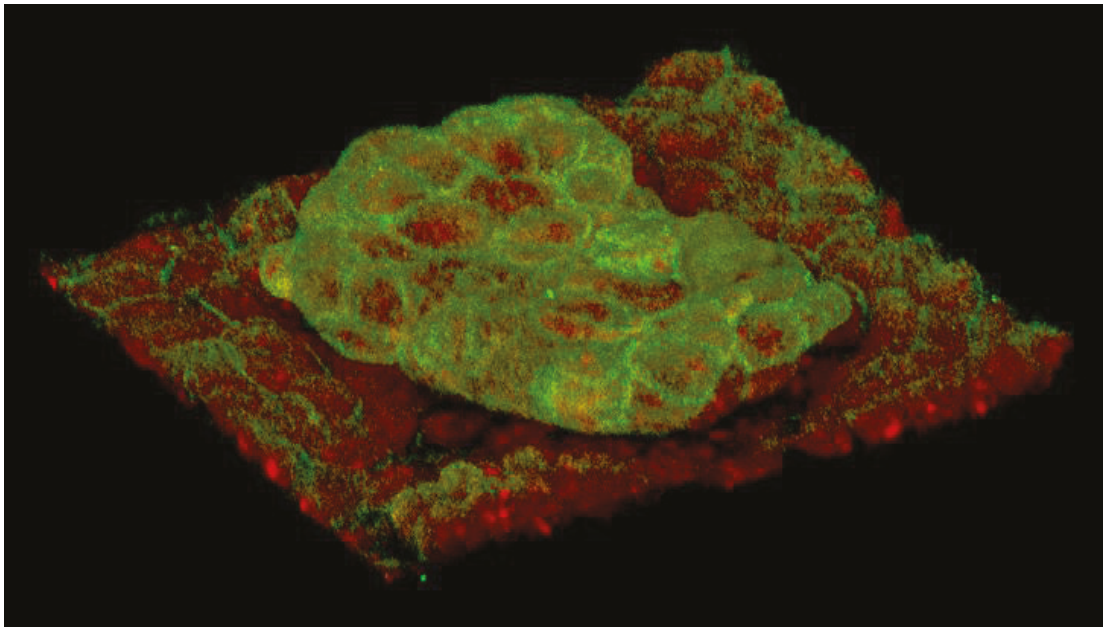
Copper Homeostasis in Human Mammary Epithelial Cells



by

David Freestone, BSc.

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University

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Publications Arising From This Project

The data presented in this thesis has been submitted or is currently in preparation for publication.

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Freestone, D., Michalczyk, A., Jakab, M., Patterson, D., Howard, D., de Jonge, M., and Ackland, M.L. ATP7A overexpression disrupts the balance of copper homeostasis in cultured PMC42-LA mammary epithelial cells, manuscript in preparation.

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Abstract

Adequate amounts of copper in milk are critical for normal neonatal development, however the mechanisms regulating copper supply to milk have not been clearly defined. The mammary gland is an unusual organ as it expresses three copper efflux molecules; ATP7A, ATP7B and ceruloplasmin (Cp). Analysis of mammary epithelial cells provides the opportunity to investigate their interactions. PMC42-LA cell cultures representative of the resting (no hormones), lactating (2.7 ng/mL β-estradiol, 157 ng/mL progesterone, 1 µg/mL dexamethasone 0.6 µg/mL insulin and 200 ng/mL prolactin) and suckled mammary epithelium (similar to the lactating model, but with 800 ng/mL prolactin) were used to investigate the role of copper transporting proteins in copper homeostasis and to provide insights into the delivery of copper to milk.

The addition of copper to cultured mammary epithelial cells reduced the presence of CTR1 at the cell membrane and increased the dispersion of ATP7A and ATP7B throughout the cells. The dispersion of ATP7A correlated with an increase in ATP7A at the plasma membrane, while ATP7B was not detected at the membrane in any conditions. These responses to extracellular copper suggest that the cells balance cellular copper levels by reducing uptake and increasing efflux. Elevated copper conditions also augmented the level of the ferroxidase form of Cp which is important in iron metabolism. When exposed to high copper levels, the cells did not effectively efflux all of the absorbed copper and it accumulated within the cells. Expression of metallothionein (MT) correlated with the intracellular copper levels, consistent with its role as a metallochaperone

Lactational hormones increased the presence of CTR1 at the plasma membrane. This correlated with an increased level of copper within the cells. ATP7A responded becoming more dispersed, consistent with its role in trafficking copper across the basolateral membrane when copper levels are elevated. This may be a defensive mechanism to protect a suckling infant from receiving too much copper in the milk. However Cp levels are also increased which would promote copper secretion into the milk. Increased transport of copper across the membranes due to changes in protein expression and localisation may enable the cells to easily and quickly redirect copper where required, whether across the apical membrane into the milk or across the basolateral membrane away from the milk. Syncrotron X-ray fluorescence and AAS revealed that the organoids grown in culture representing the mammary gland lobules, secrete copper into the lumen of the organoid. This effect was enhanced in the presence of lactational hormones, reflecting the *in vivo* conditions of the mammary gland lobules during lactation.

ATP7A, a copper efflux protein, was overexpressed in PMC42-LA cells to obtain insight into its role in lactation. The impact of this homeostatic disturbance on the other copper transporting molecules was investigated. The overexpression of ATP7A caused an increase in CTR1 and ATP7B in the presence of hormones, responses thought to counteract additional movement of copper across the basolateral membrane. Increasing CTR1 and ATP7B levels stimulates the movement of copper towards the apical membrane for secretion into the milk, thus preventing the secretion of copper

deficient milk. Copper levels also regulated ATP7B expression. Most notably in the ATP7A overexpressing cells treated with lactational hormones, ATP7B levels were reduced in response to high copper levels. This is thought to regulate the efflux of copper across the apical membrane into the milk. The levels of β2-microglobulin (β2M), a protein involved in iron uptake, increased in response to ATP7A overexpression, as did Cp, indicating that overexpressing ATP7A not only disturbed copper homoestasis, but also impacted upon iron pathways.

A soluble secreted form of Cp as well as membrane bound glycosylphosphatidylinositol-linked Cp (GPI-Cp) were detected in the PMC42-LA cells. The GPI-Cp expression was unaffected by copper or lactational hormones, but increased with ATP7A overexpression similar to the secreted Cp. Additionally, an interaction between ATP7B and Cp was confirmed, suggesting that Cp receives its copper from ATP7B. No interaction between ATP7A and Cp was identified.

In summary, these data highlight the important roles that CTR1, ATP7A, ATP7B, Cp and MT play in maintaining copper homeostasis. Additionally, they demonstrate the interactions between ATP7A, ATP7B and Cp, that control copper efflux across the apical and basolateral membranes to ensure safe copper levels within the cells and to provide an adequate supply of copper into milk.

CHAPTER 1

Introduction

1.1 Copper in biological systems

Copper is a trace element required for cellular growth and development. It is incorporated into a number of enzymes with a multitude of biochemical functions (Linder, 1991). Copper binding proteins facilitate strict regulation of copper homeostasis by taking up, distributing, storing or effluxing copper as required (La Fontaine and Mercer 2007). Imbalances to copper homeostasis can have dramatic effects upon cellular functions. Excess copper can be detrimental due to its redox properties. Unbound ionic copper can produce free radicals in cells, leading to damage of lipids, proteins, RNA and DNA. Excess copper can also displace other metal ions from their enzymes leading to altered function or inactivation of these enzymes. Copper deficiency can lead to the inactivation of copper dependent enzymes. For example, inactivation of copper dependent enzymes like Cu/Zn superoxide dismutase, a free radical scavenger, can lead to increased oxidative damage. Whereas disruption to cytochrome c oxidase due to copper depletion can reduce ATP levels which can affect metabolic activity (Pena et al., 1999).

1.2 Copper in humans

Copper is available to humans through diet, with seafood, meat and seeds being the richest sources of copper and to a lesser extent fruit and vegetables (Linder and Hazegh-Azam, 1996). Approximately 2.5 mg per day is the recommended daily intake with about 75% being absorbed by the intestine (Lutsenko et al., 2007a, Vonk et al., 2008), however it has been shown that copper uptake is regulated according to body copper concentration. Mammals absorb more when their body is deficient and absorb less when the body concentrations are sufficient or in excess (Linder,

1991, Turnlund, 1998). Copper is absorbed across the intestinal epithelium into the portal blood stream where it is transported to the liver (Kuo et al., 2006, Monty et al., 2005, Nose et al., 2006, Ravia et al., 2005). Before entering the portal bloodstream, it is bound to copper chaperone proteins including albumin and α2-macroglobulin (Linder et al., 1998, Moriya et al., 2008). The delivery of copper to these chaperones is critical to prevent the toxic effects of its ionic form. Copper is absorbed from the portal vein by the liver which is the main regulator of copper homeostasis in the body. When required, copper is transferred from liver cells into the main circulatory system of the body (Linder et al., 1998). Excess amounts of copper are excreted from the body via the biliary canaliculus into the bile and expelled in the faeces (Linder et al., 1998, Terada et al., 1999). Bile is the main form of copper excretion from the body with 98% of total copper lost through this pathway (Wijmenga and Klomp, 2004). Before entering the blood stream from the liver, copper is incorporated into ceruloplasmin (Cp) (Linder and Hazegh-Azam, 1996, Linder et al., 1998, Terada et al., 1998). While circulating around the body, copper is absorbed by cells and incorporated into cuproenzymes, enabling their activity (Vonk et al., 2008). Copper within cells exists bound to metallochaperones like metallothionein, glutathione, ATOX1, CCS and COX17 (Amaravadi et al., 1997, Culotta et al., 1997, Ferreira et al., 1993, Freedman et al., 1989a, Freedman et al., 1989b, Glerum et al., 1996, Klomp et al., 1997, Lin et al., 1997, Sturtz et al., 2001). Binding copper to chaperones prevents copper from creating reactive oxygen species which cause damage to cells.

In humans, there are a number of enzymes and cellular processes that require copper for efficient function. Copper dependent enzymes include: lysyl oxidase, which is involved in the cross linking collagen and elastin; tyrosinase, which helps in the formation of melanin; cytochrome c oxidase, which is involved in the electron transport chain; peptidylglycine α amidating monooxygenase, which is involved in the processing of neuropeptides; superoxide dismutase which scavenges free radicals; cross-linkase, which cross-links keratin; dopamine β hydroxylase, which helps to produce catecholamine; and finally Cp, which is a multicopper ferroxidase, oxidises Fe (II) to Fe (III) (Giraud et al., 1992, Kagan and Li, 2003, Matoba et al., 2006, Osaki et al., 1966, Peterson et al., 1998, Richardson et al., 1975, Rush and Geffen, 1980, Sato and Gitlin, 1991, Tsukihara et al., 1995). Disruption to the function of these enzymes as a result of copper deficiency can have dire consequences.

1.3 Copper deficiency

Copper homeostasis must be tightly regulated as imbalances in copper levels can have a great impact on the quality and length of life. In a copper deficient environment, the activity of copper dependent enzymes is reduced or completely abated. Impeded function of lysyl oxidase and cross-linkase, prevents the cross linking of collagen, elastin and keratin, causing problems in connective tissue leading to loose skin and joints, and brittle hair. Deficient activity of tyrosinase leads to hypopigmentation of the skin. Dopamine β hydroxylase deficiency can lead to hypothermia, hypotension, dehydration and somnolence as a result of hypothalamic imbalance. Widespread disturbances caused by malfunctioning peptide hormones are thought to

occur due to copper-dependent peptidylglycine α amidating monooxygenase inactivity, however specific details are yet to be elucidated. Copper depleted Cp has an impact upon iron metabolism and can lead to anemia (Mercer, 2001, Peterson et al., 1998).

It has been demonstrated that the average western diet contains less copper than the recommended daily average. This is leading to a higher prevalence of copper deficiency in the community (Klevay, 2011, Sánchez et al., 2010). Copper deficiency has been associated with diseases including abdominal aortic aneurysm, Alzheimer's disease, coronary artery disease, coronary heart disease, ischemic heart disease and osteoporosis, all of which are becoming more prevalent in modern society. It is not yet clear whether copper deficiency has a role in the etiology of the disorders (Klevay, 2011). However, compelling evidence suggests that copper deficiency influences the onset of osteoporosis, cardiomyopathy and hypertension (Allen et al., 1982, Chiplonkar et al., 2004, Conlan et al., 1990, Kawada et al., 2006, Klevay, 1998, Saari et al., 2007).

Menkes disease is an X-linked recessive genetic disorder that causes general copper deficiency in the body (Danks et al., 1972, Menkes et al., 1962). The disease can be identified by a number of symptoms including kinky/steely depigmented hair, loose skin with no pigment, loose skeletal joints, neurological degeneration and retardation, seizures and growth retardation (Danks et al., 1972, Menkes et al., 1962). Patients usually show symptoms of Menkes disease by 2-3 months of age and if untreated, can cause death by the age of three years. Patients also exhibit low copper

concentrations in the blood as well as low Cp levels (Danks et al., 1972, Menkes et al., 1962). The milder form of Menkes disease displays similar symptoms, however the neurological degeneration is far less severe or nonexistent which prolongs the life of the patient (Danks,1988, Kaler et al., 1994, Procopis et al., 1981).

Menkes disease arises due to various mutations in the *ATP7A* gene (Chelly et al., 1993, Mercer et al., 1993, Vulpe et al., 1993). Over 200 different mutant variations have been described, including missense and nonsense mutations as well as insertions, deletions and splice site mutations (Tumer et al., 1999, Hsi and Cox, 2004). The effects of mutations can range in severity and three types of Menkes disease have been described; classical Menkes disease, mild Menkes disease and Occipital Horn Syndrome (Danks, 1995). Mutations to the *ATP7A* gene can inhibit the function of the protein by preventing ATP binding due to an altered protein structure. ATP7A is then incapable of functioning and so copper cannot be transported through the protein pore. Nonsense mutations can cause *ATP7A* transcription to terminate prematurely, preventing the full transcription of the protein. Mutations can also cause improper trafficking and mislocalisation of the ATP7A protein. *ATP7A* mutations can cause the protein to be unresponsive to copper and remain in the *trans*-Golgi network (TGN) when intracellular copper concentrations increase. In other instances ATP7A can remain near the plasma membrane when copper levels are reduced. Some mutations can also cause ATP7A to mislocalise to the endoplasmic reticulum. Trafficking irregularities and mislocalisation all inhibit copper efflux from cells. In milder forms of Menkes disease, ATP7A retains some capacity to function although

it is less effective at effluxing copper (Bertini and Rosato, 2008, de Bie et al., 2007, Kim et al., 2003, Voskoboinik et al., 2003).

Copper deficiency caused by Menkes disease arises due to copper homeostatic disturbances in the intestine. Intestinal enterocytes take up copper from the digested food in the gut, however in Menkes disease patients, copper transfer to the bloodstream is inhibited (Kodama et al., 1999, Kuo et al., 2006, Monty et al., 2005, Nose et al., 2006, Ravia et al., 2005). This causes a build up of copper in the enterocytes leading to toxicity, however the rest of the body remains copper deficient as there is little copper in the blood (Cheng and Bayliss, 2008). No effective cure is available for Menke's Disease, however the addition of copper into the bloodstream in the form of copper-histidine can impede the progression of symptoms and prolong the patient's life expectancy. Patients with residual ATP7A activity have been shown to respond better to treatments than those without. Copper supplementation also appears to be more effective against the development of neurological symptoms than some of the other symptoms like the skeletal defects (Komada et al,. 2012, Tumer and Moller, 2010).

1.4 Copper toxicity

In addition to the problems encountered when copper levels are deficient, excess copper can also be detrimental to health. Ionic copper is toxic and in high doses can produce reactive oxygen species that cause damage to cells. Long term exposure to high levels of reactive oxygen species in humans can lead to diseases like cancer, stroke, myocardial infarction and diabetes (Karthikeyan et al., 2011). Acute exposure to high levels of copper induces

nausea, vomiting, diarrhea, and abdominal pain (Araya et al., 2001). There have also been instances where infants exposed to drinking water contaminated with high copper levels from copper piping, developed liver failure, occasionally leading to their death (Schramel et al., 1988, Walker, 1999). Copper can also enter the body via routes other than ingestion. It was shown that vineyard workers using copper based fungicide sprays had up to 10 fold high levels of copper in buccal cells compared to non exposed controls (Thompson et al., 2012). Lifestyle factors can also contribute to an elevation in copper levels in the body. Studies have shown that smoking and also low levels of activity leads to increased copper levels (Sánchez et al., 2010).

Copper toxicity is not limited to environmental factors. Wilson's disease is an autosomal recessive genetic disorder which leads to copper toxicity. Clinical markers of Wilson's disease include a build up of copper in the cornea of the eye (Kayser-Fleischer rings), excess copper in the urine, low copper levels in the bile, low Cp levels, neurological symptoms like depression and schizophrenia, and hepatic symptoms like jaundice, hepatitis or cirrhosis (Mak and Lam, 2008).

Wilson's disease symptoms can manifest themselves at various ages, ranging from two to over forty years of age (Mercer, 2001). Wilson's disease is only fatal if left untreated, usually as a result of liver failure (Ala et al., 2007). Organ damage can be avoided if the disease is identified early and treatment is administered (Mak and Lam, 2008). While the greatest impact of copper toxicity is on the liver, other organs like the brain are also subjected to

damage. Neurological symptoms are present in Wilson's disease approximately 50% of the time (Vonk et al., 2008). Damage occurs mainly due to release of reactive oxygen species caused by copper accumulation (Sokol et al., 1994). Copper toxicity in the liver arises from the inability of ATP7B to excrete copper into the biliary canaliculus (Roelfosen et al., 2000, Schaefer et al., 1999).

The abnormal copper status of Wilson's disease patients is due to mutations in the *ATP7B* gene which inhibit ATP7B's ability to bind ATP and also to traffic properly causing an accumulation of copper in the liver. Over 250 different genetic mutations of the *ATP7B* gene are known, most of which are missense mutations, although some are the result of small insertion or deletion mutations (Cox and Moore, 2002, Hsi and Cox 2004). Mutations that disrupt trafficking can render the protein unresponsive to copper causing it to remain localised in the TGN in all copper conditions. Mutations can also cause the mislocalisation of ATP7B where it remains in the endoplasmic reticulum (de Bie et al., 2007, Forbes and Cox, 1998, Forbes and Cox, 2000). All known mutations cause varying degrees of Wilson's disease. The severity and type of symptoms differ greatly, even between patients, with the same genetic mutation (Riordan and Williams, 2001).

Copper is incorporated into Cp from ATP7B in the liver and then the copper loaded Cp is dispersed into the blood for transport around the body (Linder and Hazegh-Azam, 1996, Linder et al., 1998, Terada et al., 1998). When ATP7B is defective, less copper is incorporated into Cp (Scheinberg and Gitlin, 1952, Sternlieb et al., 1973, Terada et al., 1998). This leads to reduced

Cp levels, as copper free Cp degrades more rapidly than the copper loaded form (Gitlin and Janeway, 1960, Matsuda et al., 1974, Sternlieb et al., 1961).

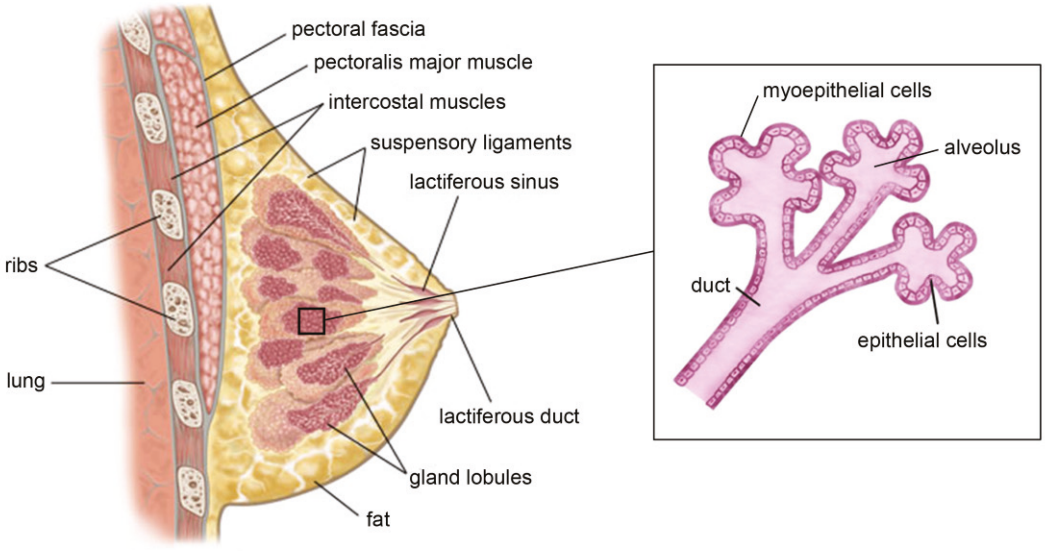
1.5 The mammary gland

The maternal milk secreted from the mammary gland is an important source of nutrition for neonates. The mammary gland is made up of lobular structures surrounded by adipose and connective tissue. Each lobule is made up of 10-100 alveoli which are lined with epithelial cells surrounding a central lumen. Mammary epithelial cells are polarised cells with microvilli on the apical membrane. Junctions between cells include adherens junctions, desmosomes, tight junctions and gap junctions (Lanigan et al., 2007, Ozzello, 1971, Tannenbaum et al., 1969). Mammary epithelial cells have certain distinct features including the presence of lipid granules, large secretory vacuoles, swollen endoplasmic reticulum and basally located nuclei (Ackland et al., 2001, Ackland et al., 2003, Monaghan et al., 1985, Whitehead et al., 1983a, Whitehead et al., 1983b). The epithelial cells of the alveoli are surrounded by contractile myoepithelial cells, which are themselves surrounded by a basement membrane (Lanigan et al., 2007). The alveoli are connected to ducts, which converge to form larger ducts that transport the milk to the nipple, enabling an infant to feed (Geddes, 2007) (Fig 1.1).

Until pregnancy, mammary epithelial cells remain dormant. Hormonal changes in pregnancy play an important role in modification of the mammary gland, initiating growth and proliferation of both the ducts and the alveoli, increasing the number of secretory cells available (Lamote et al., 2004).

Figure 1.1 Structure of the mammary gland

A cross-section of the human breast shows the lobes, ducts and surrounding tissues. The magnified image shows the arrangement of cells at the terminal region of the ducts called the alveolus.



Adapted from Encyclopedia Britannica

Hormones including estrogen, progesterone, prolactin and insulin are important in ductal development and alveolus formation (Hovey et al., 2002). By mid-pregnancy, the mammary epithelial cells begin to secrete colostrum (Geddes, 2007). Withdrawal of estrogen and progesterone from the circulation helps initiate the secretion of milk at the beginning of lactation (Neville et al., 2002, Pang and Hartmann, 2007, Rillema, 1994). Prolactin and insulin are important in the synthesis and secretion of milk from mammary epithelial cells (Hovey et al., 2002). Insulin may also play a role in providing nutrients to the mammary gland which are then used for milk production (Neville et al., 2002). Suckling initiates the release of oxytocin from the posterior pituitary gland, causing contraction of the myoeplithelial cells surrounding the mammary gland. This contraction squeezes the milk out of the lobules and along the ducts. Suction from the infant also aids the removal of milk from the mammary gland (Geddes, 2007). Glucocorticoids similar to dexamethasone also perform roles in the differentiation of mammary glands and the induction of lactation (Neville et al., 2002).

1.6 Copper in lactation

Copper levels in the milk must be adequate for the growth and development of the infant shortly after birth. The importance of copper in milk is clearly illustrated by the toxic milk (*tx*) mutant mouse model, in which a defective ATP7B protein impedes the supply of copper into the dam's milk causing the suckling pups to develop symptoms of copper deficiency leading to their death after approximately two weeks (Michalczyk et al., 2000, Rauch, 1983). Copper levels fluctuate greatly in the mother's blood during gestation and lactation compared to normal levels. While unaffected by dietary intake, milk

copper levels change with progression of lactation (Dorea, 2000). During lactation, the rate of copper uptake by the mammary gland is increased 20-fold in rats (Donley et al., 2002). Milk copper concentrations are highest after birth (0.4–0.6 mg/L in humans, 3.2-6.4 mg/L in rodents), but decrease as lactation progresses both in humans (0.2–0.3 mg/L) and rodents (0.9-2.1 mg/L) (Friel et al., 1999, Kelleher and Lonnerdal, 2006, Linder et al., 1998, Lonnerdal, 1996, Perrone et al., 1993, Salmenpera et al., 1986, Tsymbalenko et al., 2009, Wooten et al., 1996). Copper transport and secretion into milk has also been shown to be stimulated by suckling (Kelleher and Lonnerdal, 2006). During lactation, it is vital that copper is secreted into milk at a rate that will not expose the suckling infant to excess or deficient copper levels, thus causing harm to the neonate, which could affect its growth and development.

1.7 Copper homeostasis in mammary epithelial cells

Copper homeostasis in the mammary gland is controlled by a small number of influx and efflux proteins. The current model suggests that CTR1 brings copper from the blood plasma into the mammary epithelial cells across the basolateral membrane (Kelleher and Lonnerdal, 2003, Kelleher and Lonnerdal, 2006, Llanos et al., 2008). ATOX1 then delivers the copper to either ATP7A or ATP7B in the TGN (Banci et al., 2007, Banci et al., 2009, Strausak et al., 2003, Tanchou et al., 2004, van Dongen et al., 2004). The efflux of copper from the cells is accomplished by translocating ATP7A to the basolateral membrane where it is released back into the maternal circulation (Kelleher and Lonnerdal, 2006, Llanos et al., 2008), while ATP7B traffics copper towards the apical membrane where it is thought to deliver copper to

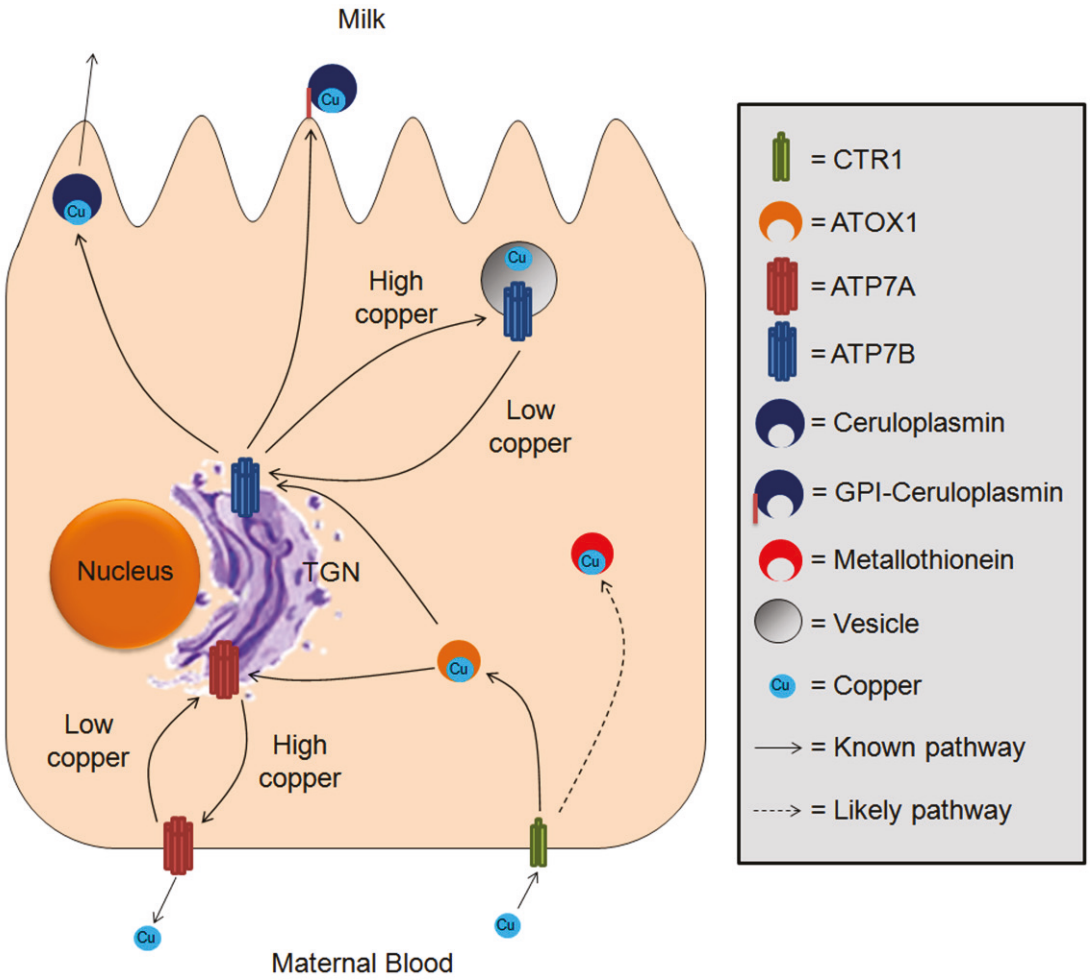
Cp which is then secreted into the milk (Kelleher and Lonnerdal, 2006, Platonova et al., 2007, Tsymbalenko et al., 2009). It is also likely that ATP7B delivers copper to the membrane bound glycosylphosphatidylinositol anchored form of Cp (GPI-Cp). The majority of copper found in milk is bound to the Cp secreted from the mammary epithelial cells (Platonova et al., 2007, Tsymbalenko et al., 2009). Metallothionein, a copper storage protein, sequesters excess copper in the cell when copper levels are elevated (Mehta et al., 2006, Sutherland and Stillman, 2011, Urani et al., 2003) (Fig 1.2).

1.8 CTR1

In humans CTR1 has not been linked to any diseases, although when knocked out in mice, it has been shown to be embryonically lethal, indicating the importance of the protein in copper homeostasis (Kuo et al., 2001, Lee et al., 2001). CTR1 is a 190 amino acid protein (Zhou and Gitschier, 1997) which, once glycosylated, is approximately 35 kDa in size (Klomp et al., 2002). CTR1 has three transmembrane domains with the *N*-terminus on the extracellular side and the *C*-terminus on the cytosolic side (Klomp et al., 2003). Electron crystallography showed that three CTR1 proteins bind together to form a radially symmetrical conical shaped channel which is widest on the cytoplasmic side. It has been shown that the *N*-terminal region is important in accepting Cu (II) from the external environment. Once bound, Cu (II) is converted to Cu (I) enabling it to be transported passively through the protein channel to the *C*-terminal region that makes copper available to copper chaperones like ATOX1. The yeast ATOX1 homologue protein ATX1 was demonstrated to accept copper (I) from the *C*-terminal domain of CTR1 (de Feo et al., 2009, Eisses and Kaplan, 2005, Ferruzza et al., 2000, Hill and

Figure 1.2 The molecular interactions maintaining copper homeostasis in mammary epithelial cells

This is a schematic diagram of copper transporting proteins in mammary epithelial cells. The diagram shows the localisation of proteins with regard to the apical and basolateral membranes of mammary epithelial cells. Arrows identify known and possible pathways of copper movement through cells.


Milk
Cu
High copper
Low copper
Nucleus
TGN
Low copper
High copper
Maternal Blood
= CTR1
= ATOX1
= ATP7A
= ATP7B
= Ceruloplasmin
= GPI-Ceruloplasmin
= Metallothionein
= Vesicle
= Copper
= Known pathway
= Likely pathway

Link, 2009, Howell et al., 2010, Lee et al., 2002b, Puig et al., 2002, Xiao and Wedd, 2002).

The mechanism by which copper influx is regulated is quite contentious. A number of research groups suggested that CTR1 binds copper and the protein is then internalised into cellular vesicles and degraded (Guo et al., 2004a, Guo et al., 2004b, Holzer and Howell, 2006, Holzer et al., 2004a, Liu et al., 2007, Petris et al., 2003), on the other hand it has been proposed that the mechanism could be due to internalisation and recycling of the CTR1 complex back to the membrane (Molloy and Kaplan, 2009), while another group has demonstrated CTR1 degradation without internalisation (Safaei et al., 2009). Finally, it has even been reported that there is no degradation of the protein nor removal from the cell membrane in response to copper and that there may be another mechanism in place that regulates the influx of copper (Eisses et al., 2005, Wu et al., 2009).

1.9 ATOX1

ATOX1 is a small 7 kDa protein, classified as a copper chaperone. It is most commonly known for its role in delivering copper to either of ATP7A or ATP7B in the TGN, where its interaction with the metal binding domains of these ATPases has been confirmed (Banci et al., 2007, Banci et al., 2009, Strausak et al., 2003, Tanchou et al., 2004, van Dongen et al., 2004). There have only been a small number of studies analysing ATOX1 responses to copper and these studies showed ATOX1 to be unaffected (Andersen et al., 2007, Gambling et al., 2004). Most research on ATOX1 was carried out on *ATOX1* knockout mice. ATOX1 has proven to have a great impact upon

mouse survival, with only 55% of *ATOX1* -/- mice surviving past weaning. The low rate of survival is due to the impact ATOX1 has on regulating copper efflux. Without ATOX1, copper efflux is restricted as copper does not reach ATP7A or ATP7B which are able to remove excess copper from cells. As a result, these mice showed symptoms of copper deficiency, similar to Menkes disease, including restricted growth, loose skin, hypopigmentation and seizures (Hamza et al., 2001, Hamza et al., 2003). ATOX1 may have additional functions including the unbiquitination of CTR1, targeting it for degradation. It is also thought that ATOX1 may have a role in the nucleus as a transcription factor, influencing cellular proliferation (Itoh et al., 2008, Muller and Klomp, 2009). ATOX1 expression levels have also been shown to be tightly linked with SOD3 expression, indicating it may have some role in relieving oxidative stress (Jeney et al., 2005).

1.10 ATP7A

ATP7A is a P-type ATPase that exports copper across the basolateral membrane in most cell types except hepatocytes (Vulpe et al., 1993). Human ATP7A protein has a number of important functional regions. These include eight transmembrane domains that cross the lipid bilayer, forming a cylindrical channel. The *N*-terminal region contains six metal binding sites that are specific for copper binding prior to its transport through the pore formed by the trans-membrane domains. One of the cytoplasmic loops also contains an ATP binding domain which provides energy for the copper ions to be moved through the protein pore once ATP is bound (Lutsenko and Kaplan, 1995).

The localisation of ATP7A within cells is influenced by copper (Petris et al., 1996). The mechanism behind ATP7A trafficking is not clear; however it is evident that this process is important in maintaining copper homeostasis of the cell. Basal and low copper conditions in non-polarised cells cause ATP7A to reside in the TGN where it can receive copper from ATOX1. ATP7A makes copper available to copper dependent enzymes like lysyl oxidase, where it is incorporated into the enzyme structure enabling it to function (Kagan and Li, 2003, Tchaparian et al 2000). Most intracellular copper is recycled and not often excreted back into the blood from cells unless in excess (Tapiero et al., 2003). Exposure to high copper concentrations induces movement of ATP7A from the TGN towards the plasma membrane of the cell. This response is indicative of a protein that exports or facilitates the export of excess copper (Ackland et al., 1997, La Fontaine et al., 1998a, La Fontaine et al., 1998b, Petris et al., 1996). Copper excretion was proposed to occur by two methods, exocytosis, which involves ATP7A moving copper into vesicles and the vesicular transfer of copper across the plasma membrane, or by ATP7A incorporating itself into the membrane and directly transporting copper out of the cell. (Cobbold et al., 2003, Lane et al., 2004, Nyasae et al., 2006, Petris et al., 1998, Petris and Mercer, 1999, Strausak et al., 1999, Voskoboinika et al., 1998).

Experiments in non-polarised cells show trafficking of ATP7A to all parts of the plasma membrane of the cell, however trafficking is more complicated in polarised cells. ATP7A has been detected in many types of epithelial cells which have an apical side (which faces the lumen) and a basolateral side (which faces the basement membrane). Limited research has been

performed on ATP7A localisation in polarised cells; however based on the vectorial transport of copper, it is hypothesised that it would reside on the basolateral side of the epithelium (Greenough et al., 2004, Hardman et al., 2007, Llanos et al., 2008).

ATP7A has been observed in epithelial cells of lactating and non-lactating mammary gland tissue (Ackland et al., 1999, Kelleher and Lonnerdal, 2006, Llanos et al., 2008). It was demonstrated that ATP7A trafficking is induced during lactation with the protein residing in the TGN in the non-lactating mammary epithelial cells and dispersing towards the plasma membrane during lactation (Ackland et al., 1999). ATP7A was shown to be clearly visible at the basolateral membrane of lactating mammary epithelial cells (Kelleher and Lonnerdal, 2006, Llanos et al., 2008). These data fit the current model, in which copper enters the mammary epithelial cells via CTR1 and is delivered to ATP7A or ATP7B in the TGN. ATP7A is then able to make copper available for other copper dependent enzymes like lysyl oxidase, or efflux any unnecessary copper back across the basolateral membrane to the maternal blood stream, thus protecting a suckling infant from obtaining excessive amounts of copper. One study also observed ATP7A to localise towards the apical membrane in response to suckling (Kelleher and Lonnerdal, 2006). This does not fit with the current model which proposes the ATP7B and Cp are the key components involved in delivering copper across the apical membrane into milk. Unless ATP7A is also involved in delivery of copper to Cp in situations where demand is very high. It has been shown previously in the brain and in macrophages that ATP7A can be used to

deliver copper to Cp when ATP7B is not present (Barnes et al., 2005, White et al., 2009a).

1.11 ATP7B

ATP7B is a P-type ATPase found predominantly in the liver. In the mammary gland, placenta, kidney, brain, heart and lungs, ATP7B is expressed along with ATP7A (Bull et al., 1993, La Fontaine and Mercer, 2007). ATP7B has two main functions in the liver; firstly, it moves copper into the biliary canaliculi, where it is incorporated into bile and excreted from the body via the faeces (Linder et al., 1998, Terada et al., 1999); secondly, ATP7B in liver cells loads Cp with copper which enables copper to be safely transported around the body in the blood (Linder and Hazegh-Azam, 1996, Linder et al., 1998, Terada et al., 1998).

ATP7B has 67% similar amino acid sequence to ATP7A, and like ATP7A has eight transmembrane domains which form a pore through which ATP catalyses the transport of copper. ATP7B also contains six copper binding sites at the *N*-terminal region like ATP7A (Bull et al., 1993). Similar to ATP7A, ATP7B's trafficking is also dependent on intracellular copper concentrations. ATP7B resides in the TGN under basal and copper depleted conditions where it predominantly facilitates the incorporation of copper into Cp (Cater et al., 2004, Forbes and Cox, 2000, Hung et al., 1997, La Fontaine et al., 1998b, Linder and Hazegh-Azam, 1996, Linder et al., 1998, Terada et al., 1998). When copper concentrations increase, ATP7B begins to traffic towards the cell membrane with the intention of transporting excess copper out of the cell. ATP7B does not travel directly to the membrane. It was

proposed that ATP7B delivers copper to vesicles, possibly late endosomes, which then efflux copper from the cell via exocytosis (Cater et al., 2006, Harada et al., 2005). In polarised cells, ATP7B traffics towards the apical side of the cells, as opposed to ATP7A which traffics towards the basolateral side (Bartee and Lutsenko, 2007, Guo et al., 2005, Kelleher and Lonnerdal, 2006). It was suggested that the 63 amino acid sequence that is present in ATP7B and not in ATP7A is responsible for the trafficking of ATP7B in the opposite direction to ATP7A (Guo et al., 2005).

The importance of ATP7B in delivering copper from the mammary epithelium into milk has been clearly demonstrated with the toxic (*tx*) mutant mouse model. The *tx* mouse has a defective *ATP7B* gene, rendering the protein incapable of proper function. The dams produce copper deficient milk causing suckling neonates to develop symptoms of copper deficiency. This can lead to death of the pups within two weeks (Michalczyk et al., 2000, Rauch, 1983). Immunolocalisation analysis of ATP7B in resting and lactating mammary epithelial cells shows a similar pattern to ATP7A. Trafficking of ATP7B appeared to increase in response to lactation (Michalczyk et al., 2008). ATP7B has demonstrated apical localisation, consistent with ATP7B's role in delivering copper to milk (Kelleher and Lonnerdal, 2006), however it is not known whether ATP7B directly transports copper across the membrane or facilitates efflux via vesicles, but it has been shown that Cp contains a high proportion of copper in milk and so it is likely that ATP7B plays a vital role in loading copper onto Cp for secretion into milk. (Platonova et al., 2007, Tsymbalenko et al., 2009).

1.12 Ceruloplasmin

Ceruloplasmin (Cp) has been found expressed in the liver, macrophages, lymphocytes, choroid plexus, yolk sac, placenta, uterus, testis, lung, heart, kidney and the mammary gland; from which it had been found to be secreted into the milk (Cerveza et al., 2000, Tsymbalenko et al., 2009, Wooten et al., 1996, Yang et al., 1990). Cp is known to have a number of roles in the human body which may include copper transport. Cp is secreted from the liver and circulates in the blood carrying up to 60-95% of serum copper. The remaining copper in the blood is bound to albumin and low molecular weight complexes (Cerveza et al., 2000, Jaeger et al., 1991, Lockhart and Mercer, 1999, Wooten et al., 1996). A lack of disturbance to copper levels in systemic tissues in the genetic disorder aceruloplasminemia and also in *Cp* knockout mice, suggested that Cp is not an essential component of copper distribution around the body (Meyer et al., 2001, Miyajima, 2003, Miyajima et al., 1987, Waggoner et al., 1999). However, other research has suggested it is a key component in copper uptake in rats and cultured cells (Bae and Percival, 1994, Campbell et al., 1981, Davidson et al., 1994, Lee et al., 1993, Linder, 1991, Linder et al., 1998, Owen, 1971, Percival and Harris, 1990). Contrary to this, aceruloplasminemia patients and *Cp* knockout mice have demonstrated disturbances to iron metabolism. When Cp is absent, iron accumulates in liver, pancreas, retina and brain (Patel et al., 2002, Xu et al., 2004). This occurs because copper bound Cp has ferroxidase activity and is able to oxidise Fe (II) to Fe (III) (Osaki et al., 1966, Sato and Gitlin, 1991). Converting the more toxic Fe (II), to the more inert Fe (III) form of iron, not only protects cells from oxidative damage, it also allows iron to bind to proteins like transferrin (which only binds Fe (III)), making it mobile and

available for circulation around the body (Chen et al., 2003, Collins et al., 2010, Hellman and Gitlin, 2002, Rathore et al., 2008). There are also other transferrin like proteins, which are similar in structure and also bind Fe (III), one of which, lactoferrin, interacts with Cp and is found to be a major iron transporter in the milk (Dorea, 2000, González-Chávez et al., 2009, Ha-Duong et al., 2010, Kawakami et al., 1990, Sabatucci et al., 2007, Sokolov et al., 2006, Zakharova et al., 2000). Cp is involved in the uptake (Chang et al., 2007) and efflux (de Domenico et al., 2007, Harris et al., 1999) of iron from cells. Along with its ferroxidase capability, Cp has also been shown to oxidise amines and low-density lipoproteins (Boivin et al., 2001, Mazumder and Fox, 1999, Tapryal et al., 2009).

The *tx* mutant mouse which has a defective *ATP7B* gene has around 22% of the normal level of copper in the secreted milk (Rauch, 1985). It has been shown that Cp contains up to 85% of copper in milk and so it is likely that ATP7B is responsible for the incorporation of all the copper into Cp (Platonova et al., 2007, Tsymbalenko et al., 2009). It has been suggested the remaining fraction of copper in milk may be bound to other soluble copper dependent enzymes like superoxide dismutase (Tsymbalenko et al., 2009). Copper levels in milk have been shown to decline with the progression of lactation; a trend followed by Cp suggesting that milk copper levels are dependent on to the Cp levels in the milk (Albera and Kankofer, 2009, Cerveza et al., 2000, Keen et al., 1982, Platonova et al., 2007, Tsymbalenko et al., 2009).

An alternate, membrane bound form of Cp (GPI-Cp) is also expressed in most tissues (Banha et al., 2008, Fortna et al., 1999, Kono et al., 2010, Mittal et al., 2003, Mostad and Prohaska, 2011, Patel and David, 1997) and is linked to the plasma membrane by a glycosylphosphatidylinositol anchor (GPI-Cp) (Patel and David, 1997). GPI-Cp has also been implicated in iron oxidation and efflux from cells, though not in iron uptake (Chang et al., 2007, Jeong and David, 2003, Kono et al., 2006a).

1.13 Metallothionein

There are four known types of metallothionein; MT-1, MT-2, MT-3 and MT-4 (Margoshes and Vallee, 1957, Quaife et al., 1994, Thirumoorthy et al., 2011, Uchida et al., 1991). MT-1 and MT-2 are ubiquitously expressed, while MT-3 and MT-4 have been found in CNS and squamous epithelial cells respectively (Masters et al., 1994, Miles et al., 2000, Quaife et al., 1994). MTs have roles in metal homeostasis and detoxification, free radical scavenging and protection against oxidative stress, and have also been implicated in antiapoptotic defence mechanisms and neuroprotection (Penkowa et al., 2006, Sato and Bremner 1993). MTs have 20 cysteine residues which are important in the binding of metals (Sutherland and Stillman, 2011). MT-1 and MT-2 have been shown to bind and respond to metal ions of cadmium, copper and zinc (Nielson et al., 1985, Vasak and Meloni, 2011). MT binds cadmium more strongly than zinc, while copper has a higher affinity to MT than both cadmium and zinc, however zinc has greater influence upon MT expression levels (Sutherland and Stillman, 2011, Urani et al., 2003). Research has also shown that MT can bind to metals like antimony, bismuth, cobalt, indium, iron, lead, mercury, nickel, osmium,

palladium, platinum, rhenium, rhodium, silver, technetium and thallium (Good and Vasak, 1986, Morelock et al., 1988, Nielson et al., 1985, Ngu et al., 2010).

1.14 Copper and iron homeostatic association

Copper and iron homeostasis are linked, with the levels of one metal being affected inversely to the other. In cultured hepatocarcinoma cells, it was shown that increases in copper levels, decreased iron levels (Fosset et al., 2009). The opposite effect was observed in rats fed Cu deficient diets where iron levels were raised significantly in the liver and intestine, however the spleen, kidney and bone had less iron (Chen et al., 2006). Supporting the copper-iron relationship in living organisms, rats fed high iron diets had lower copper levels than rats fed low iron diets (Gambling et al., 2004). These changes may be mediateld by altered Cp activity. Increasing copper concentration allows ferroxidase active Cp to metabolise more iron from Fe (II) to Fe (III) enabling it to become more mobile and be effluxed from cells; the opposite applies during copper deficiency (Chen et al., 2006, Fosset et al., 2009).

1.15 Transferrin receptor, β2-microglubulin and iron uptake

The main iron uptake pathway in cells is via transferrin receptor 1 (TfR) which binds iron-loaded transferrin and is then endocytosed (Bhatt et al., 2010, Enns, 2001). β2-microglobulin (β2M) plays an important role in TfR iron uptake by creating a complex with HFE, a protein that binds to TfR and inhibits its capacity to accept transferrin. When β2M interacts with HFE it prevents its inhibitory action on TfR allowing this protein to freely bind

transferrin (Bhatt et al., 2009, Bhatt et al., 2010, Enns, 2001, Vecchi et al., 2010). The stability of β2M is affected by the presence of Cu (II) (Deng et al., 2006). In situations where copper status is altered, β2M protein can be affected. It has been shown that cells overexpressing β2M increase their uptake of iron (Waheed et al., 2002).

1.16 PMC42-LA mammary epithelial cell line

In this study, the PMC42-LA cultured human mammary epithelial cell line was used to analyse copper transporting proteins and the mechanisms controlling copper homeostasis. The PMC42 cell line was originally derived from the pleural effusion fluid of a woman who had metastatic breast cancer. The PMC42 cells were originally grown in suspension and also as a surface monolayer of cells. Eight different morphological cell types were identified in the culture which included undifferentiated stem cells, spindle shaped cells, syncytium forming cells, large cells and small epithelial cells with differing intracellular characteristics. These observations led the authors to believe that the PMC42 cells originated from a tumorous breast stem cell (Whitehead et al., 1983a, Whitehead et al., 1983b). Initial characterisation of the PMC42 cells also revealed that they contain receptors for estrogen, progesterone and dexamethasone and that the cells responded to all of these hormones. Other mammary epithelial cells lines have failed to respond to these hormones giving the PMC42 cells line a distinct advantage in analysing the effects of lactation which requires hormonal stimulation (Whitehead et al., 1984).

A major difference between lactating mammary tissue and cultured mammary cells is the degree of cellular differentiation. Previous studies showed that PMC42-LA cells grown on plastic have some breast-specific features including lipid granules, swollen endoplasmic reticulum and large secretory vacuoles (Whitehead et al., 1983a, Whitehead et al., 1983b). However, when grown on EHS matrix coated permeable membranes, PMC42-LA cells form organoids similar to the alveolar structures of the mammary gland where cells are arranged around a central lumen. These cells develop a phenotype more representative of differentiated luminal epithelial cells, including the presence of tight junctions and microvilli, basally located nuclei and the presence of lipid granules (Ackland et al., 2001). PMC42-LA cells also express proteins found in milk. The iron transporting milk protein lactoferrin has been detected in cells grown on plastic and on EHS matrix coated membrane. However, upon differentiation, the milk protein β-casein is also expressed, but only in the presence of lactational hormones. β-casein has often been used as a marker of fully differentiated lactating mammary epithelial cells. Lactation is induced with the addition of estrogen and progesterone for three days following a week of culturing. These hormones are then removed and replaced with prolactin, insulin and dexamethasone (Ackland et al., 2001, Ackland et al., 2003, Michalczyk et al., 2008). As in physiological conditions, the withdrawal of estrogen and progesterone has a role in initiating the milk secretion process, while prolactin, insulin and glucocorticoids like dexamethasone are key to maintaining the synthesis and secretion of milk from mammary epithelial cells (Hovey et al., 2002, Neville et al., 2002, Pang and Hartmann, 2007, Rillema, 1994).

1.17 Hypotheses

The following research is based on hypotheses that copper transport proteins which are the subject of the thesis, have specific roles in the movement of copper into the milk during lactation. These hypotheses are based on data describing the functions of the molecules in other cells/tissues. CTR1 is proposed to be the first point of contact, bringing copper into the mammary epithelial cells from the blood stream. Once inside the cells ATOX1 is hypothesised to deliver copper to the TGN to the ATPases ATP7A and ATP7B. ATP7A and ATP7B are copper efflux proteins predicted to export copper from the mammary epithelial cells across the basolateral and apical membranes respectively. ATP7B is proposed to deliver copper to the mulit-copper ferroxidase ceruloplasmin, which is then secreted via the apical membrane into the milk. The milk is then ejected from the mammary gland to feed a suckling infant. Lactational hormones including estrogen, progesterone and prolactin that are known to regulate milk section from the mammary gland and these hormones are hypothesised to influence the movement of copper into mammary epithelial cells and then into the secreted milk.

1.18 Aims

The following research, analyses the activity of CTR1, ATOX1, ATP7A, ATP7B and Cp in the PMC42-LA cell line in relation to copper transport in lactation. To commence the project, the PMC42-LA cells were used in their non-differentiated state. The cells were then differentiated and lactation was induced by treatment with lactational hormones to attain a more accurate understanding of the mechanisms involved *in vivo*. In addition to the non-

hormonally treated resting model and the hormonally treated lactation model, a third model was created with a higher concentration of prolactin to simulate suckling conditions. To gain further insight into copper transport homeostasis, ATP7A was overexpressed in PMC42-LA cells. Copper transporters in both the undifferentiated and differentiated cells were investigated. The differentiated ATP7A overexpressing cells were also treated with lactational hormones to simulate lactation and suckling. These studies enabled the analysis of a potential copper depletion caused by a homeostatic disruption in a system containing three copper efflux proteins; ATP7A, ATP7B and Cp.

PMC42-LA cells were exposed to two different copper treatments. The levels used were similar to those found in the blood during lactation and non-lactational periods. This was studied to gain insight into the copper transporter responses that occur solely as a result of the variable copper levels found in the blood during these periods. Additionally, copper chelators were also used to identify the responses due to copper deficiency.

The overall objective of this research was to increase the current knowledge of the roles that copper transporting proteins play in delivering copper to milk. By observing changes of the proteins involved in copper uptake and efflux from mammary epithelial cells in response to altered copper status and fluctuations in hormonal levels, a greater understanding of the processes involved in maintaining an adequate, non-toxic supply of copper into milk which provides nutrition for suckling neonates, can be obtained.

1.19 Approach

A key approach to this research is the use of the the PMC42-LA mammary epithelial cells that can be induced to form a 3D organoid. This organoid is a model representative of the lobules of the mammary gland. The treatment of cells with lactational hormones induces the secretion of the milk protein β-casein, a key marker of lactation. Hormone-treated PMC42 cells exposed to hormones thus provide a model of the lactating human gland. Treatment of cells with variable copper levels will allow the replication of the physiological conditions encountered in the mammary gland during lactation. mRNA and protein analysis and protein localisation of the copper transporting proteins by qRT-PCR, Western blot and fluorescence confocal microscopy respectively will provide crucial information about the function of the copper transporters. Trace metal levels and localisation determined by atomic absorption spectroscopy and X-ray fluorescence microscopy will provide insights into the link between copper molecules and transport of copper. This approach will enable analysis of the behaviour of the copper transporting proteins during lactation compared to non-lactational periods, identifying how changes in copper homeostasis affects the intracellular copper levels and the potential effects this may have on the copper levels secreted into milk. It will also shed light on interactions between the different copper transport molecules within the mammary gland.

CHAPTER 2

Materials and Methods

2.1 Plasmid preparation and cell transfection

A previously developed ATP7A overexpression plasmid, pCMB344 (Ke et al., 2006) was used to transfect PMC42-LA using polyethylenimine solution (Shin et al., 2005). Specifically, 20 µg plasmid DNA was diluted in 150 mM NaCl to a concentration of 10 µg/mL. 4.2 mg/mL polyethylenimine (MR 2000) pH 5.0 was added to DNA/NaCl solution to a final concentration of 0.813 mg/mL. The mixture was vortexed for 10 sec and incubated for 30 min at room temperature. The mixture was added to 15 mL RPMI 10% FBS and added overnight to a 75 cm^2 culture flask containing PMC42-LA cells that were 75% confluent. Transfected cells were selected using 500 µg/mL G418 (Sigma-Aldrich; Sydney, Australia) in RPMI 10% FBS medium. Fresh medium with antibiotics were added every three days.

2.2 Cell culture

The breast adenocarcinoma cell line PMC42-LA, a variant of the PMC42 line, originally derived from a pleural effusion (Whitehead et al., 1983a), was cultured in RPMI 1640 culture media (Thermo Fisher Scientific; Melbourne, Australia) supplemented with 10% FBS (Bovogen; Melbourne, Australia) at 37ºC in 5% CO_2. The cells were passaged when confluent using 0.05% trypsin-EDTA solution in PBS (Sigma-Aldrich; Sydney, Australia). Cells were grown in 75 cm^2 culture flasks (Nunc; NY, USA) for Western blot, Quantitative Real Time PCR and AAS analysis, 6-well plates (Nunc; NY, USA) for biotinylation or on glass coverslips for immunofluorescent analysis. Cells were treated with a combination of 200µM bathocuproine disulfonate (BCS) (Sigma-Aldrich; Sydney, Australia) and 200µM D-penicillamine (DPen) (Sigma-Aldrich; Sydney, Australia) to deplete the available pool of copper,

while 5 μM $CuCl_2$ (Sigma-Aldrich; Sydney, Australia) was added to simulate basal serum conditions and be used as a control treatment. 50 μM $CuCl_2$ was added to analyse the effect of surplus copper on the cells, similar to conditions reported *in vivo* during lactation. All treatments were for three days. To identify whether iron influenced Cp levels, cells were treated with 0μM (control), 100μM, 300μM, 500μM and 1000μM $FeCl_3$ for three days then processed for Western blot analysis

To induce cellular differentiation, PMC42-LA cells were grown on porous Transwell filters (BD Falcon; Sydney, Australia) coated with a thin layer of undiluted extracellular matrix gel from Engelbreth Holm-Swarm mouse sarcoma (Sigma-Aldrich; Sydney, Australia) (EHS matrix). Differentiated PMC42-LA cells were treated with lactogenic hormones to obtain a cellular model of lactating epithelia as we previously described (Ackland et al., 2001, Ackland et al., 2003, Michalczyk et al., 2008). Cells were grown for seven days and then treated with 2.7 ng/mL β-estradiol (Sigma-Aldrich; Sydney, Australia) and 157 ng/mL progesterone (Sigma-Aldrich; Sydney, Australia) for three days, followed by a three day treatment of 1 μg/mL dexamethasone (Sigma-Aldrich; Sydney, Australia), 0.6 μg/mL insulin (Sigma-Aldrich; Sydney, Australia), and 200 ng/mL prolactin (Jomar Diagnostic; Melbourne, Australia). The cells grown on the porous Transwell filters were processed for immunofluorescence or harvested for protein, mRNA or trace metal analysis. Regular suckling causes constantly elevated plasma levels of prolactin (Neville et al., 2002). To create a model representative of the mammary gland during suckling, the cells were treated the same as the lactating model except 800 ng/mL prolactin was added instead of 200 ng/mL for the final

three days. No hormones were added to the model that simulates the resting breast. Copper treatments were also carried out during the last three days as described above.

2.3 Antibodies

A polyclonal rabbit anti-human CTR1 antibody was raised to the first 98 amino acids and affinity purified using an AminoLink Plus Immobilization Kit (Thermo Fisher Scientific; Melbourne, Australia) (Hardman et al., 2006, Ke et al., 2008, Llanos et al., 2008). A polyclonal sheep anti-human ATP7A (R17) antibody was raised and affinity purified as previously described (Ke et al., 2006). Antibodies to ceruloplasmin (Dako; Melbourne, Australia – CAT #A0031), ATP7B (Saphire Bioscience; Sydney, Australia - CAT# PAB12477), Na/K ATPase (Thermo Fisher Scientific; Melbourne, Australia – CAT# sc-48345), ATOX1 (Abnova; Taipei City, Taiwan – CAT# H00000475-M01), MT (Dako; Melbourne, Australia – CAT # M0639), β2-microglobulin (Saphire Bioscience; Sydney, Australia – CAT# ab75853), transferrin receptor (Saphire Bioscience; Sydney, Australia – CAT# NB100-92243) and β-actin (Sigma-Aldrich; Sydney, Australia – CAT# A5441) were purchased commercially.

2.4 Immunocytochemistry

PMC42-LA cells, grown on coverslips or on undiluted EHS matrix coated, porous Transwell cell culture inserts and following treatment were fixed in 4% paraformaldehyde (Sigma-Aldrich; Sydney, Australia) for 10min, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich; Sydney, Australia) (3% for cells on matrix) in PBS (Amresco; Ohio, USA) for 10 min, and blocked

with 1% BSA (Sigma-Aldrich; Sydney, Australia) in PBS for 10 min (3% BSA in PBS for 1 h for cells on matrix). Primary antibody diluted in 1% BSA in PBS (ATP7A 1/4000; ATP7B 1/1000; CTR1 1/3000; Cp 1/1000) was applied to cells overnight at 4 °C. For immuno-colocalisation ATP7A antibody was added in combination with Cp antibody. Following PBS washes, a secondary antibody, AlexaFluor 488 chicken anti-rabbit (Molecular Probes; Melbourne, Australia) (ATP7B, CTR1, Cp) or AlexaFluor 488 donkey anti-sheep (Molecular Probes; Melbourne, Australia) (ATP7A) diluted 1/2000 in 1% BSA in PBS, was applied for 2hr at room temperature. AlexaFluor 488 donkey anti-sheep and AlexaFluor 594 donkey anti-rabbit were used in combination, diluted 1/2000 for immuno-colocalisation. After washing off excess secondary antibody with a 10 min PBS wash, ethidium bromide (Sigma-Aldrich; Sydney, Australia) (1 μg/mL) was added to cells for 45 sec to enable visualization of the nuclei (this was not done for immuno-colocalisation). The cells were then washed three times quickly to remove any excess ethidium bromide, followed by two more 10 min PBS washes. Coverslips and filters were then mounted on glass slides in ProLong Gold antifade reagent (Invitrogen; Melbourne, Australia). Confocal images were collected using a Leica confocal microscope system TCS SP2 (Leica; Melbourne, Australia). Image J co-localisation software was used to determine Pearson's co-localisation coefficient for co-localisation analysis. Statistical significance is indicated by $p<0.05$.

2.5 Western blot analysis – whole cells

PMC42-LA cells grown in 75 cm^2 culture flasks were collected by trypsinisation and washed three times in PBS. Lysates were prepared using

500 µL of 1% (w/v) SDS (Sigma-Aldrich; Sydney, Australia) in 10 mM Tris-HCl (pH 7.5) (Sigma-Aldrich; Sydney, Australia) lysis buffer whereas PMC42-LA cells grown on filters were collected from the Transwell cell culture inserts after exposure to 250 µL of 2% (w/v) SDS in 10 mM Tris-HCl (pH 7.5) lysis buffer for 5 min. Lysates were homogenised by repeatedly passing the lysate through a 21-gauge needle followed by sonication (15 pulses, 40% power output, 30% duty cycles; Microson XL2000 Ultrasonic Cell Disruptor, Misonix, Farmingdale, NY, USA) on ice in 1x EDTA-free inhibitor cocktail (Roche Diagnostics; Melbourne, Australia). The total protein content of cell extracts was measured using the BCA Protein Assay Kit (Pierce; Melbourne, Australia) calibrated against BSA standards. 60 µg of lysates were fractionated by SDS-PAGE on 7.5% (ATP7A, ATP7B, Cp, TfR), 12% (CTR1) or 15% (ATOX1, MT, β2M) gels and transferred to nitrocellulose membranes (Whatman; Dassal, Germany). Membranes were then stained with 0.1% Ponceau S (Sigma-Aldrich; Sydney, Australia). Ponceau S stain was removed with 0.1% NaOH (Sigma-Aldrich; Sydney, Australia). After 1 h blocking in 5% (w/v) milk powder in TBS at room temperature, membranes were exposed overnight at 4 °C to primary antibody diluted in TBS: ATP7A 1/4000; ATP7B 1/1000; CTR1 1/3000; Cp 1/1000; ATOX1 1/500; MT 1/50; β2M 1/10000; TfR 1/500. Membranes were washed four times for 10 min in TBS and antibodies were detected using 1/2000 dilution of horseradish peroxidase–conjugated goat anti-rabbit (ATP7B, CTR1, Cp, β2M, Tfr), donkey anti-sheep (ATP7A) or goat anti-mouse (ATOX1, MT) secondary antibody (Millipore; Melbourne, Australia) in TBS for 45 min at room temperature. After removal of excess secondary antibody, membranes were rinsed twice in TBS for 10 min and then twice more in TBS with 0.1% Tween

20 (Sigma-Aldrich; Sydney, Australia). Proteins were detected by enhanced chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate; Millipore; Melbourne, Australia) and a LAS-3000 FujiFilm Lumino-Image Analyzer (Fuji Photo Film; Tokyo, Japan). Blots were stripped for 5-10 min using Reblot Plus Strong (Millipore; Melbourne, Australia) solution and then reprobed with β-actin monoclonal antibody diluted 1/5000 or Na/K ATPase antibody diluted 1/1000 followed by goat anti-mouse secondary antibody to determine the protein loading for lysates and biotinylation respectively. Densitometry to quantify results was performed using Fuji Film Multi Gauge V3.0 computer software, and ratios for protein levels were calculated relative to β-actin or Na/K ATPase.

2.6 Western blot analysis – cell secretions

At the completion of each cell culture experiment, the remaining growth medium was collected and spun at 10,000 xg for 10 min to remove any suspended cells. The supernatant was collected and protein concentration estimated as was done for the cells. 60μg of protein was fractionated by SDS-PAGE on a 7.5% gel and transferred to nitrocellulose membranes (Whatman; Dassal, Germany) and probed for Cp as stated above. Images of the Ponceau S stain were taken using LAS-3000 FujiFilm Lumino-Image Analyzer and were used to determine the loading of the growth medium. Densitometry to quantify results was performed using Fuji Film Multi Gauge V3.0 computer software, and ratios for protein levels were calculated relative to the Ponceau S image.

2.7 Quantitative real time PCR (qRT-PCR)

PMC42-LA cells grown in 75 cm^2 culture flasks were collected by trypsinisation and washed three times in PBS. PMC42-LA cells grown on filters were collected from the Transwell cell culture inserts using 1mL of 5mg/mL dispase (Gibco; Melbourne, Australia) dissolved in PBS. The dispase was left on the cells for 45 min until all cells detach from the EHS matrix. Cells were processed using an RNeasy Mini Kit (QIAGEN; Melbourne, Australia) following manufacturer's protocol. Total RNA was purified by DNase1 treatment following the manufacturer's protocol of the DNA-free kit (Ambion; Melbourne, Australia). RNA concentrations were determined using a Nanodrop 100 spectrophotometer (Thermo Fisher Scientific; Melbourne, Australia). Once quantified, RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Melbourne, Australia) following the manufactures protocol. Quantitative Real Time PCR was performed using 7500 Real Time PCR system (Applied Biosystems; Melbourne, Australia) with 20 µl sample volumes containing 8 µl SYBR green mastermix (Applied Biosystems; Melbourne, Australia), 0.3 µM of both forward and reverse primers (Table 1) and 20 ng cDNA. The PCR reaction was performed at 50ºC 2 min, 95ºC 10 min (1 cycle), 95ºC 15 sec, 60ºC 1 min (40 cycles). Relative mRNA expression was determined by comparing the expression of the target gene (*ATP7A, ATP7B, CTR1, Cp, ATOX1, β2M*) with housekeeping control (*β-actin*) using the equation $2^{-\Delta\Delta CT \pm SD}$, where ΔΔCT was the difference between the control ΔCT and the treatment ΔCT.

Table 1.1 Real Time PCR Primers

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
ATP7A	GCTACCTTGTCAGACAACGAATGAC	TCTTGAACTGGTGTCATCCCTTT
ATP7B	CCAGCAAAGCCCTTGTTAAGTT	CACGAAAGCCAATTTCCTCAAT
CTR1	ACTCCAACAGTACCATGCAACCT	AGTTCCACATTCTTAAAGCCAAAGTAG
Ceruloplasmin	GACACCATCAGAGTAACCTTCCATAA	CCCCAATCGGCTCAATACTG
ATOX1	GCTTGGAGGAGTTAAGTATGACATTG	TTTCTTCAGGGTTGCAAGCA
β2-microglobulin	AGCGTACTCCAAAGATTCAGGTT	ATGATGCTGCTTACATGTCTCGAT
β-actin	GACAGGATGCAGAAGGAGATTACT	TGATCCACATCTGCTGGAAGGT

2.8 Reverse transcription PCR (RT-PCR)

RNA was extracted from PMC42-LA cells, purified and converted to cDNA as specified for qRT-PCR. PCR was performed using GoTaq Flexi Kit (Promega; Sydney, Australia) as per manufacturer's instructions, using primers and thermocycling conditions as specified by Banha et al. (2008). PCR products were separated on a 1.5% Agarose (Amresco; Ohio, USA) in TAE buffer.

2.9 Surface biotinylation

Cells were grown in 6-well plates or on EHS matrix coated Transwell filters. Following treatment cells were placed on ice and allowed to cool to 4ºC. All buffers were also cooled to 4ºC before use. Growth medium was removed and cells were washed three times with PBS wash buffer supplemented with 0.1 mM $CaCl_2$ (Sigma-Aldrich; Sydney, Australia) and 1.0 mM $MgCl_2$ (Sigma-Aldrich; Sydney, Australia). EZ-Link Sulfo-NHS-SS-biotin (Pierce; Melbourne, Australia) was dissolved in biotinylation buffer (10 mM triethanolamine (Sigma-Aldrich; Sydney, Australia), pH 7.5, 2 mM $CaCl_2$, 150 mM NaCl (Sigma-Aldrich; Sydney, Australia)) to a concentration of 0.77 mg/mL. 400 µl of Biotin was added per well and incubated for 25 min on ice while rocking. Biotin was removed and cells were washed once with quenching buffer (PBS with 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$, 100 mM Glycine (Sigma-Aldrich; Sydney, Australia)), then incubated in quenching buffer for 20 min on ice while rocking. This step was repeated once more. Lysis buffer (1% Triton X-100, 150 mM NaCl, 5mM EDTA, 1x protease inhibitor cocktail (Roche; Melbourne, Australia), 50 mM Tris-HCl, pH 7.5) was added and incubated for 2 h at 4ºC, rotating end-over-end. The lysate was then centrifuged at 10,000 xg for 10

min. The supernatant was transferred along with 0.15 volume of Streptavadin-agarose (Pierce; Melbourne, Australia) into a fresh tube and incubated at 4ºC overnight, rotating end-over-end. Samples were then centrifuged at 500 xg for 5 min and the supernatant aspirated using a 23-guage needle bent into a z shape near the tip. The resin was then washed with lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5) three times, salt wash buffer (0.1% Triton X-100, 500 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5) two times and no salt buffer (10 mM Tris, pH 7.5) once. The resin was then centrifuged after the final wash at 10,000 xg for 10 min. Protein was eluted from the streptavadin agarose resin using 40 µl of SDS-PAGE lysis buffer (1% SDS, 300 mM DTT, Tris-HCl, pH 7.5). Protein was quantified and separated by SDS-PAGE.

2.10 Co-immunoprecipitation

PMC42-LA cells grown in 75 cm^2 culture flasks were collected by trypsinisation and washed three times in PBS. The pellet was incubated in 100 µl of lysis buffer (150 mM NaCl, 1.0% NP-40 (Sigma-Aldrich; Sydney, Australia), 50 mM Tris (pH 8.0)) on ice for 30 min with occasional mixing. The sample was then spun at 10,000 xg for 10 min at 4ºC. The supernatant was transferred to a fresh tube and combined with 2 µg of ATP7B or ATP7A antibody and incubated for 1 h on ice. The mixture was then combined with 100 µl of Immobilized protein G (Pierce; Melbourne, Australia) and rotated end-over-end at room temperature for 2 h. Protein G beads were pelleted from solution by centrifugation at 1000 xg for 1 min and washed 5 times for 5min with 1mL lysis buffer at room temperature. Proteins bound to protein G beads were removed by heating to 85ºC for 10 min in 40 µl 1% (w/v) SDS in

10 mM Tris-HCl (pH 7.5) lysis buffer with 100 mM DTT. The sample was then spun at 10,000 xg for 1 min and the supernatant collected for processing by Western blot and probed with the Cp antibody.

2.11 Atomic absorption spectroscopy

PMC42-LA cells grown as stated above in 75 cm^2 culture flasks were collected by trypsinisation and washed three times in PBS. PMC42-LA cells grown on EHS matrix coated Transwell filters were collected using 1 mL of 5 mg/mL dispase and washed three times in PBS. Cells were then centrifuged at 600 xg for 5 min and resuspended in 2 mM EDTA in PBS (pH 8.0) and treated for 30 min at 37ºC. Cells were washed three times in PBS then collected, lysed and quantifed as for Western blot analysis. Lysates were then dried for seven days at 85ºC. Once dry, samples were resuspended in 500 µl concentrated Suprapur Nitric Acid (Merck; Melbourne, Australia), vortexed briefly then incubated at room temperature for 1 h. The samples were then incubated at 65ºC for 3 h, opening the tube cap every half an hour to release the build up of gas. Following the incubation, samples were vortexed briefly while still hot and then 2.5 mL (1 mL for Transwell filters) ElgaQ water was added, vortexed again and then left until ready for AAS analysis. Samples were analysed by electrothermal graphite furnace atomic absorption spectrometry, using a Varian GTA 100 spectrophotometer (Varian; Melbourne, Australia).

2.12 Liquid-chromatography mass-spectrometry

BCS/DPen treated cells grown in a 75 cm^2 flask of cells were pelleted, lysed and sonicated as specified previously, then separated on 7.5% and 12%

SDS-PAGE gels. Gels were stained with Coomassie Brilliant Blue R-250 (Biorad; Sydney, Australia) (0.1% Coomassie, 50% Methanol (Merck; Melbourne, Australia), 10% acetic acid (Merck; Melbourne, Australia)) for liquid-chromatography mass-spectrometry (LCMS) or transferred to nitrocellulose and processed by Western blot to confirm immunoreactivity of bands. Coomassie stained bands were excised from the gel in 1mm cubes and destained with 10 mM ammonium bicarbonate (Sigma Aldrich; Sydney, Australia) in 50% acetonitrile (Sigma Aldrich; Sydney, Australia). Sample was shaken on a variable speed mixer for 10 min. This process was repeated three times until the gel was visibly less stained. Reducing buffer (10mM DTT (Sigma Aldrich; Sydney, Australia), 10 mM ammonium bicarbonate) was added to gel and incubated at 60ºC for 1 h. Reaction mixture was cooled to room temperature then reducing buffer was discarded. Alkylation buffer (50 mM iodoacetamide (Sigma Aldrich; Sydney, Australia), 10 mM ammonium bicarbonate) was added to sample and incubated at room temperature for 1 h. The gel was washed with 10 mM ammonium bicarbonate for 10 min on variable speed mixer and then the liquid was removed from the gel and discarded. This wash was repeated twice more. Acetonitrile (100%) was added to the gel and incubated for 10 min at room temperature on a variable speed mixer to dehydrate the gel. The acetonitrile treatment was repeated twice more. Samples were left to air dry for 5 min. Samples were rehydrated with sequencing grade trypsin (Sigma Aldrich; Sydney, Australia) (0.02 µg/µl trypsin, 10 mM ammonium bicarbonate, 10% acetonitrile) and incubated at 37ºC for 6-10 h. The supernatant was collected and mixed with 2% formic acid (Sigma Aldrich; Sydney, Australia), shaken and then spun down. Extraction buffer (50% acetonitrile, 2% formic acid) was added to the gel and

shaken for 30 min on a variable speed mixer. The liquid containing the extracted peptides was transferred to another tube and the process repeated once more, pooling the liquid. The peptide mixture was dried by vacuum centrifugation for 30 min at 35ºC. Once dried, 1% formic acid was added to the dried tube and redissolved by vortexing for 1-2 min. Samples were then processed by liquid-chromatography mass-spectrometry.

2.13 Synchrotron X-ray fluorescence

PMC42-LA cells were grown on ethanol sterilised 4mm silicon nitride windows (Melbourne Centre for Nanofabrication; Melbourne, Australia) or on EHS matrix coated Transwell filters as described in section 2.2. Following treatment, cells were rinsed twice in PBS then incubated in 2 mM EDTA in PBS (pH 8.0) for 5 min. Cells were rinsed three times in PBS and then fixed in 4% paraformaldehyde for 10 min. Cells were rinsed twice in PBS, then three times in ultrapure water (Sigma Aldrich; Sydney, Australia) and then air dried. EHS matrix coated filters with differentiated organoids were cut to an appropriate size and stuck to the silicon nitride windows with tape. At the Australian Synchrotron, the KB mirror microprobe with a spot size of 2x2 μm was used in conjunction with the 384-element silicon array Maia detector arranged in 180 degree backscatter geometry on the X-ray Fluorescence Microscopy beamline (Australian Synchrotron; Melbourne, Australia). This experiment used an X-ray energy level of 14.5KeV to excite secondary X-ray emissions from the K-shells of the first row of transition metals. Samples were scanned 10 mm from the detector, with each 2x2 μm spot being analysed for 62.5 ms. Scans covered areas of 200x200 μm. GeoPIXE version 6.3r (Library 27w32) software (CSIRO; Melbourne, Australia) was

used to compile and visualise the 2D X-ray fluorescence maps and to determine the localisation and levels of copper through traverse sections of the organoids. Quantitation of copper and iron levels was achieved by calibrating the secondary X-ray emission of a Pt standard reference foil using the GeoPIXE software.

2.14 Statistical analysis

All experiments were repeated in triplicate, and values expressed as means ± SD. Probability plots (P-P plots) were produced for all data sets to test for normal distribution. One-way analysis of variance and Tukey's honest significant difference test was used to determine statistical variation between treatments using the statistical software SPSS version 17; $p<0.05$ was considered significant.

CHAPTER 3

Copper and lactational hormones influence the CTR1 copper transporter in the PMC42-LA mammary epithelial cell culture model

3.1 Introduction

Copper is an essential dietary component. Neonates rely heavily on maternal milk for their daily dietary needs and so maintaining adequate copper levels in milk is highly important. Milk is secreted from mammary epithelial cells in the mammary gland. For copper to be secreted into the milk, it must first be absorbed into the mammary epithelial cells from the maternal bloodstream.

CTR1 plays an important role in copper uptake into cells. Thought to be the primary copper influx protein, it is important for CTR1 to be able to regulate copper uptake to protect cells from the toxic effects of copper (Eisses et al., 2005, Lee et al., 2002b). CTR1 may also be important in mammary epithelial cells in enabling adequate uptake of copper for sufficient secretion of copper into milk to nourish a suckling infant. Studies have revealed a basolateral localisation of CTR1 in the mammary gland which is consistent with the model in which CTR1 enables the uptake of copper from the maternal bloodstream into the mammary epithelial cells (Kelleher and Lonnerdal, 2003, Kelleher and Lonnerdal, 2006, Llanos et al., 2008). Little work has focussed on responses of CTR1 to lactation and suckling in humans; however the work that has been done suggests that in mice CTR1 moves towards the membrane during lactation and suckling. Increased levels of CTR1 at the plasma membrane would enable more copper to enter the mammary epithelial cells which in turn would facilitate secretion of copper into milk.

Metallothionein 1 and 2 (MT), are metal ion chaperones which play a role in the prevention of cellular toxicity by sequestering excess copper in the cells (Coyle et al., 2002, Mehta et al., 2006, Prohaska and Gybina, 2004, Sutherland and Stillman, 2011). In the mammary gland, MT could act in a protective way, not only inhibiting the toxic effects of copper by sequestering it within the mammary epithelial cells, but also preventing the secretion of excess copper into milk.

It is hypothesised that CTR1 plays a key role in the uptake of copper into mammary epithelial cells during lactation. This research aims to identify the responses to CTR1 to changing copper and lactational hormone levels, like the variations experienced between non-lactating and lactating periods, to understand how CTR1 may influence copper levels in the mammary gland. In the current study the differentiated PMC42-LA cell culture models representing the resting and lactating mammary gland have been utilised. The lactating model has also been further developed to mimic the conditions encountered by the mammary epithelial cells while an infant is suckling by increasing the concentration of prolactin in the culture. The 50 μM copper treatment also represents the maternal blood copper level in the mammary gland that is elevated in pregnancy and lactation. Using this culture system, CTR1, MT and copper levels and localisation were analysed. Total and plasma membrane bound CTR1 levels increase with the addition of hormones, which was accompanied by the accumulation of copper within the cells as well as induced secretion of copper into the lumen of the organoids. It is proposed that this mechanism may aid the movement of copper from the

maternal blood stream into milk secretions to sustain the rapid growth of a breast-fed neonate.

3.2 Results

3.2.1 CTR1 and MT protein in undifferentiated PMC42-LA cells was influenced by copper, while CTR1 mRNA remained unaltered

Undifferentiated PMC42-LA cells were grown in culture flasks and treated with varying concentrations of copper to determine the effect on CTR1 expression. A concentration of 5 μM copper was used in the growth medium to match the normal concentration of exchangeable copper in blood plasma which is in the range of 2.7 and 7.7 μM (Linder, 1991, Linder, 2002). We treated PMC42-LA cells with 50 μM copper to simulate the maternal plasma copper concentration during lactation. Copper concentrations during lactation vary widely among reports, however the upper limit is approximately 50 μM (Dorea, 2000). Copper chelators were added to determine the effect of copper deficiency in the mammary gland models. Quantitative Real Time PCR analysis determined that there was no significant difference ($p>0.05$) in *CTR1* mRNA levels between cells grown in low (BCS/DPen, 103.9 ± 19.2%), basal (5 μM copper, 100.0 ± 13.2%) or high (50 μM copper, 107.6 ± 15.1%) levels of copper (Fig 3.1a).

Analysis of protein by Western blot revealed bands at approximately 105kDa and also 35kDa which were detected with all three treatments purportedly correlating to the trimeric and monomeric forms of CTR1, however the fluctuations between the two bands in response to copper were inconsistent. This led us to question whether the 105kDa band was in fact the trimeric form of CTR1. Using liquid-chromatography mass-spectrometry (LCMS) to analyse the 105kDa protein, CTR1 could not be identified among the top 67

abundant proteins within the molecular weight range of 15-30 kDa (covering the 22 kDa native CTR1 protein). Considering the intensity of the band detected by Western blot in this region, this strongly suggests that this is non-specific immunoreactivity, rather than the CTR1 trimer, thus the following research focuses solely on the monomeric CTR1 band.

The level of the 35 kDa monomeric CTR1 form was influenced by varying copper treatments. CTR1 exhibited a significant decrease in expression in response to increasing copper levels (50 μM copper treated cells; 67.1 ± 12.2%), and a significant increase ($p<0.05$) in response to copper chelation (BCS/DPen treated cells; 239.6 ± 25.9%) compared to the 5 μM copper treated PMC42-LA cells used as a control (100.0 ± 15.1%) (Fig 3.1b).

MT sequesters copper when high copper levels are present. Western blot analysis of MT revealed it to be present only in the 50 μM copper treated cells. MT was not detected in the BCS/DPen or 5 μM copper treated cells (Fig 3.1b).

Surface biotinylation was used to determine the amount of CTR1 located at the plasma membrane when cells were exposed to varying concentrations of copper. CTR1 protein was identified at the plasma membrane in all treatments. The BCS/DPen treated cells had significantly more protein (541.4 ± 188.1%) ($p<0.05$) compared to the 5 μM copper basal conditions (100.0 ± 18.7%), which in turn was significantly higher than the levels detected in the 50 μM copper treated cells (37.6 ± 16.0%) ($p<0.05$) (Fig 3.1c).

To determine the effect of copper on CTR1 localisation, PMC42-LA cells were grown on glass coverslips and analysed by immunofluorescence confocal microscopy. CTR1 showed a granular distribution throughout the cytoplasm of cells. This staining pattern did not vary between any of the copper conditions (Fig 3.1d).

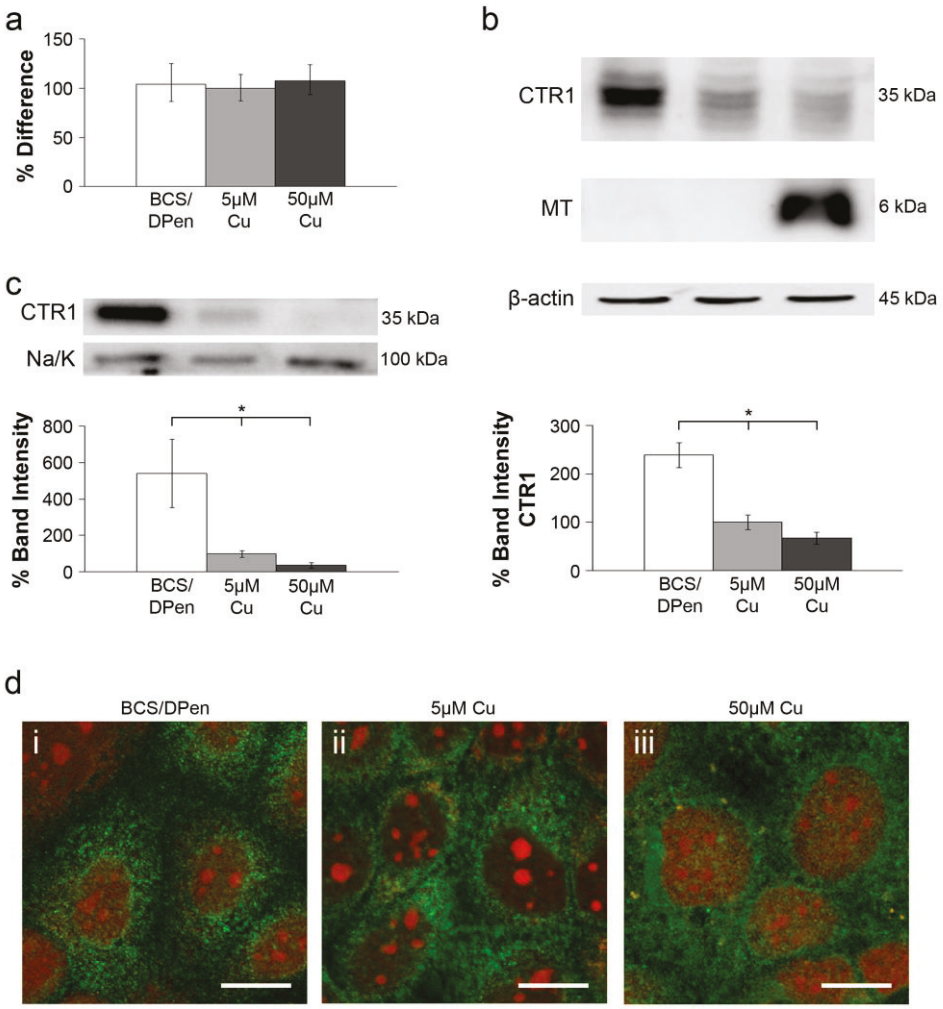
3.2.2 Intracellular copper levels increased upon exposure to high copper

Atomic absorption spectroscopy (AAS) was used to identify whether the observed changes to CTR1 protein in cells exposed to varying copper concentrations affected cellular copper accumulation. Low levels of copper were detected in BCS/DPen treated cells (32.9 ± 7.0 ng Cu/mg protein). Surprisingly, they were not significantly different from copper levels in the 5 μM copper treated cells (27.4 ± 6.7 ng Cu/mg protein), however the addition of 50 μM copper to the cells resulted in a significant rise in intracellular copper levels (586.9 ± 33.3 ng Cu/mg protein) ($p<0.05$) (Fig 3.2a).

X-ray fluorescent microscopy (XFM) was used to determine whether the changes observed in copper levels correlated with a change in distribution of copper within the cells. No copper was detected in the BCS/DPen treated cells, however a faint signal was observed in the 5 μM copper treated samples suggesting that the levels of copper in these cells was bordering the detection limit of this technique. The copper in the 50 μM treated cells was clearly visible and localised in the centre of the cells rather than the periphery, possibly in the TGN and nucleus (Fig 3.2c). Zinc was detected in

Figure 3.1 CTR1 and MT in response to copper treatments in undifferentiated PMC42 cells

(a) Relative *CTR1* mRNA levels of 50 μM copper and BCS/DPen treatments compared to that of the 5 μM copper treatment. The bar graph shows mRNA mean percentages ±SD from three independent experiments normalised against *β-actin* obtained by qRT-PCR. (b) Representative CTR1 and MT Western blot analysis of 60μg of undifferentiated PMC42-LA cell extracts. Lane 1, BCS/DPen treatment; lane 2, 5 μM copper treatment; lane 3, 50 μM copper treatment. β-actin was used as a loading control. Desitometric analysis of CTR1 band intensity are expressed as a mean percentage of arbitrary units ±SD compared to the 5 μM copper treated sample and normalised against β-actin. This figure shows representative results of three independent experiments. Asterisk indicates significant difference between samples ($p<0.05$). (c) Surface biotinylation of CTR1. The graph indicates densitometric values obtained from Western blot analysis of biotinylated CTR1 protein bands in response to BCS/DPen (lane 1), 5 μM copper (lane 2) and 50 μM copper (lane 3). Values are expressed as a mean percentage of arbitrary units ±SD compared to the 5 μM copper treated sample normalised against Na/K ATPase. Results are representative of three independent experiments. Asterisk indicates significant difference between samples ($p<0.05$). (d) Immunolocalisation of CTR1 (green) in PMC42-LA cells treated with BCS/DPen (i), 5 μM copper (ii) and 50 μM copper (iii). Nuclei were stained with ethidium bromide (red). Scale bar = 10 μm.


a
% Difference
150
100
50
0
BCS/ DPen
5μM Cu
50μM Cu
b
CTR1
35 kDa
MT
6 kDa
β-actin
45 kDa
c
CTR1
35 kDa
Na/K
100 kDa
% Band Intensity
800
600
400
200
0
*
BCS/ DPen
5μM Cu
50μM Cu
% Band Intensity CTR1
300
200
100
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*
BCS/ DPen
5μM Cu
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BCS/DPen
5μM Cu
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ii
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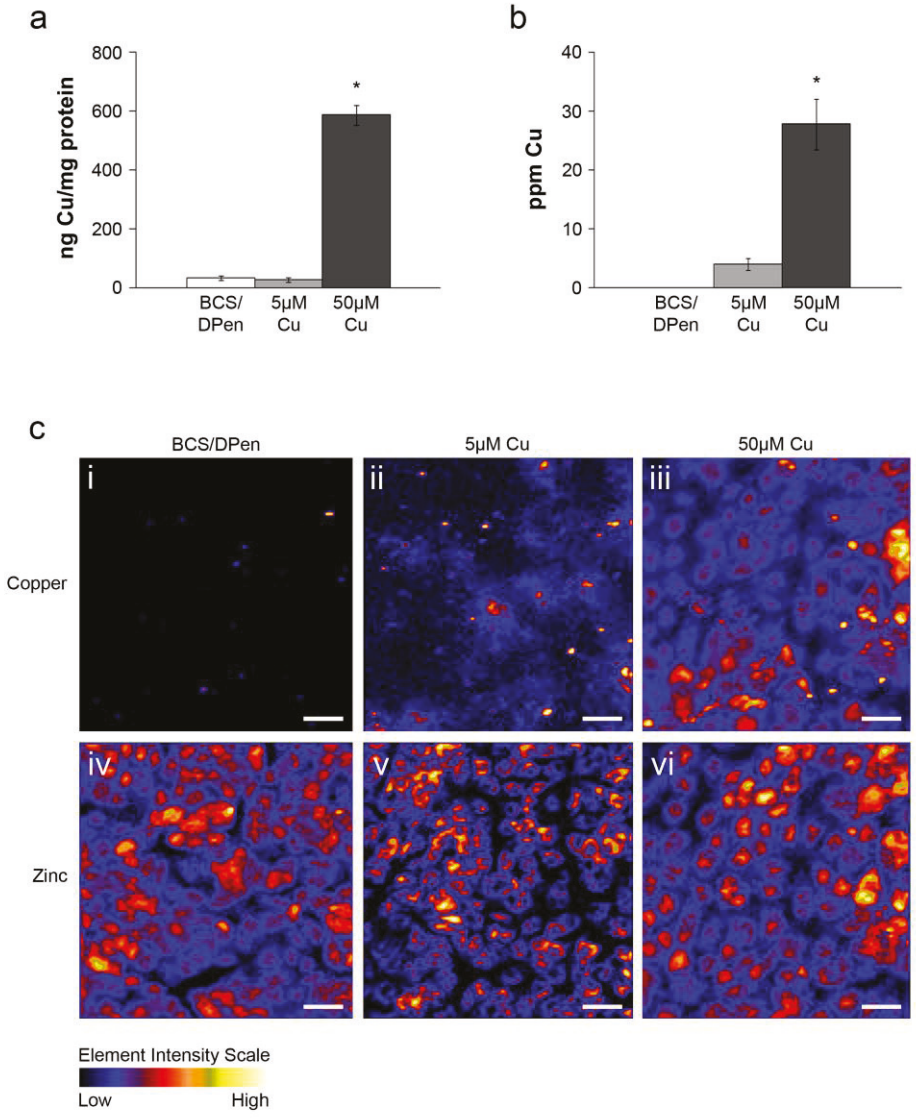
all samples and was used in this analysis to indicate the position of each cell (Fig 3.2c). Analysis of the images using GeoPIXE software revealed that the scanned cells had an average copper concentration of 27.8 ± 4.3 ppm within them when treated with 50 μM copper. This was 7 fold higher than the cells treated with 5 μM copper (4.1 ± 1.0 ppm) (Fig 3.2b). This trend reflected the data obtained by AAS analysis.

3.2.3 CTR1 mRNA levels were not influenced by copper or lactational hormones in differentiated PMC42-LA cells

In the current study, the previously characterised PMC42-LA models of resting and lactating mammary glands were used (Ackland et al., 2001, Ackland et al., 2003, Michalczyk et al., 2008). An additional model representing the sucked mammary gland was developed to further characterise the involvement of CTR1 in transporting copper into milk. For this, PMC42-LA cells were grown on EHS matrix coated Transwell filters and treated with lactational hormones together with a higher concentration of prolactin than was used in the lactating model. This mimics the physiological conditions of mammary cells during suckling where plasma prolactin levels are increased in response to mechanical stimuli and can be maintained at an elevated level if breast-feeding occurs frequently (Neville et al., 2002, Pang and Hartmann, 2007, Rhoads and Grudzien-Nogalska, 2007). The copper concentration of 50 μM represented the high maternal blood serum copper levels during early lactation, while the 5 μM copper treatment represented the non-lactating blood serum levels.

Figure 3.2 PMC42-LA intracellular copper levels in response to copper chelation and copper treatment

(a) The graph shows average values of copper levels in PMC42-LA cells treated with BCS/DPen, 5 μM copper and 50 μM copper as determined by AAS. Asterisk indicates significant difference ($p<0.05$). (b) Analysis of the XFM images using GeoPIXE software revealed the levels of copper in the cells of each treatment. (c) XFM analysis showing the localisation of copper and zinc in PMC42-LA cells treated with BCS/DPen (i and iv), 5 μM copper (ii and v) and 50 μM copper (iii and vi). The colour scale bar shows the relative levels of copper from lowest (black) to highest (white). Length scale bar = 30μm.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i
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Quantitative Real Time PCR was performed on differentiated PMC42-LA cells to identify whether mRNA levels were influenced by treatment with lactational hormones in combination with varying copper levels. No significant effect on the expression levels of *CTR1* in PMC42-LA cells was observed in the resting model (BCS/DPen – 93.5 ± 13.7%, 5 μM copper – 100.0 ± 13.4% and 50 μM copper – 93.9 ± 10.3%), lactating model (BCS/DPen – 110.9 ± 14.2%, 5 μM copper – 111.2 ± 12.5% and 50 μM copper – 98.4 ± 13.4%) or suckled model (BCS/DPen – 106.0 ± 22.5%, 5 μM copper – 106.7 ± 17.9% and 50 μM copper – 98.4 ± 13.3%) ($p>0.05$) (Fig 3.3a).

3.2.4 CTR1 and MT total protein levels altered in response to copper and lactational hormones

To identify whether protein levels reflected that of the mRNA, total protein lysates were analysed by Western blot. The observed trends in response to copper were consistent across all three models. Densitometric analysis revealed that the BCS/DPen treated samples had significantly higher CTR1 protein levels (resting – 350.5 ± 44.0%, lactating – 397.3 ± 32.4%, suckled – 439.4 ± 33.6%) than the 5 μM copper treated cells (resting – 100.0 ± 14.6%, lactating – 139.9 ± 30.7%, suckled – 170.5 ± 5.2%) and 50 μM copper treated cells (resting – 71.1 ± 11.0%, lactating – 100.7 ± 6.1%, suckled – 125.6 ± 10.7%) ($p<0.05$). While the 50 μM copper treated samples consistently proved to have less CTR1 protein that the 5 μM copper treated samples in all three models, the reduction was not statistically significant ($p>0.05$).

Comparison of the hormonal treatments in the PMC42-LA cell line revealed that the resting model cells had less CTR1 protein levels in each respective copper treatment compared to those of the lactating model. The levels in the lactating model were lower than those of the suckled model. These data show that there is statistically more CTR1 protein in each of the copper treatments of the suckled model compared to the resting model ($p<0.05$), however the differences between the resting model and lactating model and between the lactating and suckled model showed no statistical differences ($p>0.05$) despite the consistently increasing protein levels in response to elevated levels of prolactin (Fig 3.3b).

In the differentiated cells, MT was present only in the 50 μM copper treated cells similar to undifferentiated cells, however the levels also increased in response to hormones. The lactating (211.1 ± 45.7%) and suckled (325.3 ± 52.3%) models had significantly more MT than in the resting model (100.0 ± 7.8%) ($p<0.05$). MT levels were slightly elevated in the suckled model compared to the lactating model, however this difference was not significant ($p>0.05$) (Fig 3.3b). No MT was detected in the BCS/DPen or 5 μM copper treated cells for any of the three models.

3.2.5 Plasma membrane bound CTR1 levels decreased in response to copper, but increased in the presence of lactational hormones

The amount of CTR1 protein detected at the plasma membrane by biotinylation was similar to that observed in the total CTR1 protein levels (Fig 3.3b). Copper chelation increased the amount of CTR1 protein present at the plasma membrane, while the addition of copper diminished its presence. This

trend was consistent in all three models. The protein levels in response to BCS/DPen (resting – 381.4 ± 123.6%, lactating – 1705.7 ± 200.5%, suckled – 3303.6 ± 576.6%) were significantly higher than those levels detected in response to both 5 μM (resting – 100.0 ± 27.5%, lactating – 249.8 ± 53.8%, suckled – 379.6 ± 124.0%) and 50 μM copper (resting – 92.7 ± 17.1%, lactating – 210.5 ± 45.7%, suckled – 346.9 ± 106.8%) ($p<0.05$). Like the total protein levels, no statistically significant difference was observed between the 5 μM and 50 μM copper treated samples; however each model clearly showed a trend indicating there was less CTR1 protein at the membrane with the addition of 50 μM copper.

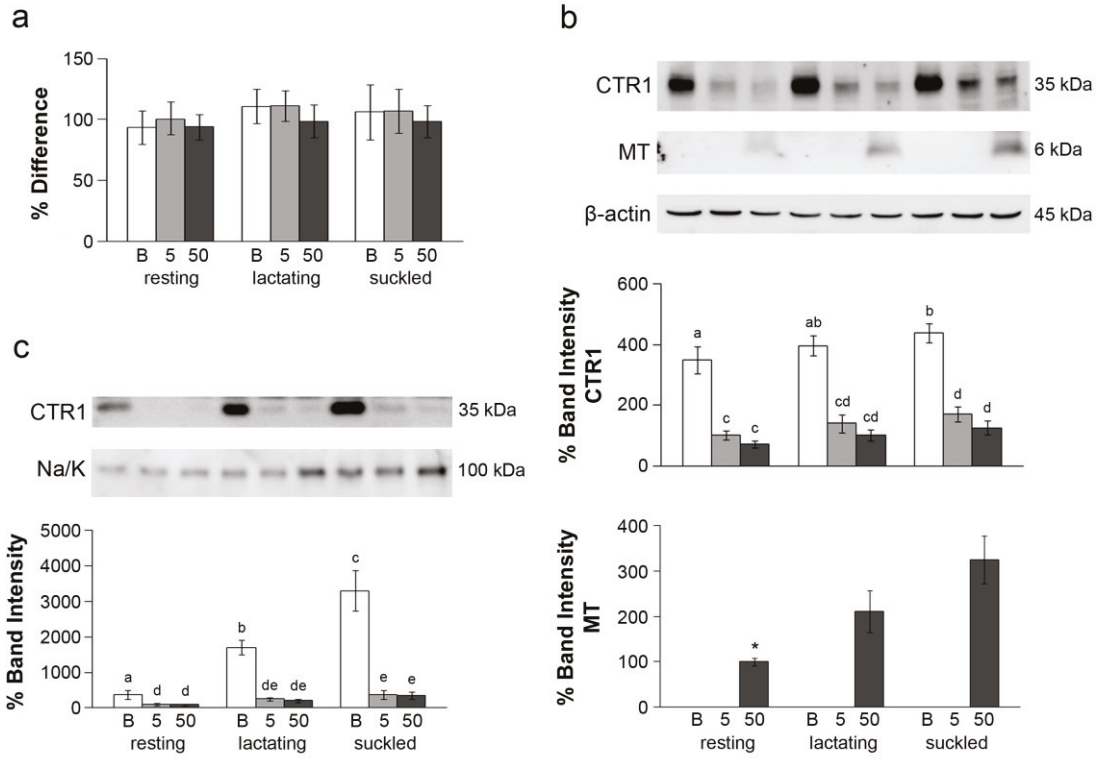
The total CTR1 protein changes observed in response to the addition of lactational hormones was similarly reflected in the levels of CTR1 protein found at the plasma membrane. Levels increased in response for hormones in each respective copper treatment with the changes between the resting and suckled models being significantly different ($p<0.05$) (Fig 3.3c). The differences between the resting and lactating values were only significant for the BCS/DPen treated cells; this was also the case when comparing the lactating and suckled models.

3.2.6 No change in CTR1 localisation was observed in PMC42-LA cells treated with copper and lactational hormones

Immunofluorescence confocal microscopy was used to visualise the localisation of CTR1 protein within the organoidal structures of the differentiated PMC42-LA cultures. Analysis revealed no obvious variations in CTR1 localisation between any of the copper or hormonally treated cultures

Figure 3.3 The copper and hormonal responses of CTR1 and MT in differentiated PMC42-LA cells

(a) Relative mRNA levels of *CTR1* in response to copper and hormones obtained by qRT-PCR. The bar graph indicates the mean percentage difference ±SD compared to the 5 μM copper treated cells of the resting model. *CTR1* mRNA levels were normalised against *β-actin*. B = BCS/DPen, 5 = 5 μM copper, 50 = 50 μM copper (b) Representative Western blots of 60μg of protein extract showing the CTR1 and MT bands in the resting breast model, lactating model and suckled model treated with BCS/DPen, 5 μM copper and 50 μM copper. The β-actin blot demonstrates relative loading of samples. The CTR1 densitometry graph bar show mean intensity of CTR1 bands expressed as a percentage of arbitrary units ±SD compared to the 5 μM copper treatment of the resting model. The MT densitometry graph shows values relative to the 50 μM copper treated resting model. Densitometric analysis of CTR1 and MT bands were normalised against β-actin. (c) This representative Western blot of extracellularly biotinylated protein extract shows the CTR1 band in the resting breast model, lactating model and suckled model treated with BCS/DPen, 5 μM copper and 50 μM copper. Densitometric analysis shows mean intensity of CTR1 bands expressed as a percentage of arbitrary units ±SD compared to the 5 μM copper treatment of the resting model. Densitometric analysis of CTR1 bands were normalised against the Na/K ATPase. All blots are representative of four independent experiments. Statistical variation is indicated by different letters and asterisk above graph bars, whereas the same letters represent no significant difference ($p<0.05$).


a
% Difference
150
100
50
0
B 5 50
resting
lactating
suckled
b
CTR1
35 kDa
MT
6 kDa
β-actin
45 kDa
% Band Intensity CTR1
600
400
200
0
a
c
ab
cd
b
d
% Band Intensity MT
400
300
200
100
0
*
c
CTR1
35 kDa
Na/K
100 kDa
% Band Intensity
5000
4000
3000
2000
1000
0
a
d
b
de
e

(Fig 3.4). The labelling of CTR1 appeared punctate and evenly distributed throughout the cell. It was difficult to determine whether this labelling was cytoplasmic, membrane or a combination of both. X-Z section analysis indicated that labelling was predominantly basolateral.

3.2.7 Intracellular copper levels of differentiated PMC42-LA cells were influenced by copper and lactational hormone treatment

Variations in copper and hormonal treatments influenced the intracellular copper levels of differentiated PMC42-LA cells. Copper levels in the resting model determined by AAS, showed no difference between the BCS/DPen (54.3 ± 23.0 ng Cu/mg protein) and 5 μM copper (61.4 ± 10.5 ng Cu/mg protein) treated cells ($p>0.05$). However the levels observed in the 50 μM copper treated cells (165.5 ± 14.7 ng Cu/mg protein) were significantly higher ($p<0.05$). This trend was replicated the lactating model (BCS/DPen – 40.4 ± 9.8 ng Cu/mg protein, 5 μM copper – 58.8 ± 16.9 ng Cu/mg protein, 50 μM copper – 520.3 ± 75.8 ng Cu/mg protein) and also the suckled model (BCS/DPen – 41.0 ± 18.4 ng Cu/mg protein, 5 μM copper – 59.0 ± 23.7 ng Cu/mg protein, 50 μM copper – 622.6 ± 78.7 ng Cu/mg protein) ($p<0.05$). Between the models, there was no difference in cellular copper accumulation in the BCS/DPen or 5 μM copper treatments ($p>0.05$). However there was a significant difference between the metal accumulation of the 50 μM copper treated resting model cells compared to the lactating and suckled models ($p<0.05$). This indicates that hormones were having an effect on the accumulation of copper in the cells, although the difference was not statistically significant between the levels of copper in the lactating and suckled models ($p>0.05$) (Fig 3.5).

Figure 3.4 CTR1 localisation in differentiated PMC42-LA cells following copper and hormonal treatment

Localisation of CTR1 (green) in the resting breast cell model in response to BCS/DPen, 5 μM copper and 50 μM copper (a, b and c). CTR1 protein localisation in the lactating breast cell model in response to BCS/DPen, 5 μM copper and 50 μM copper (d, e and f). CTR1 localisation within the suckled breast cell model in response to BCS/DPen, 5 μM copper and 50 μM copper (g, h and i). Image i of each sub-figure is a transverse section through the top of the organoid. Image ii and iii show y-z and x-z sagittal sections respectively, cutting through the centre of the organoid. Nuclei were stained with ethidium bromide (red). Scale bar = 20 μm.

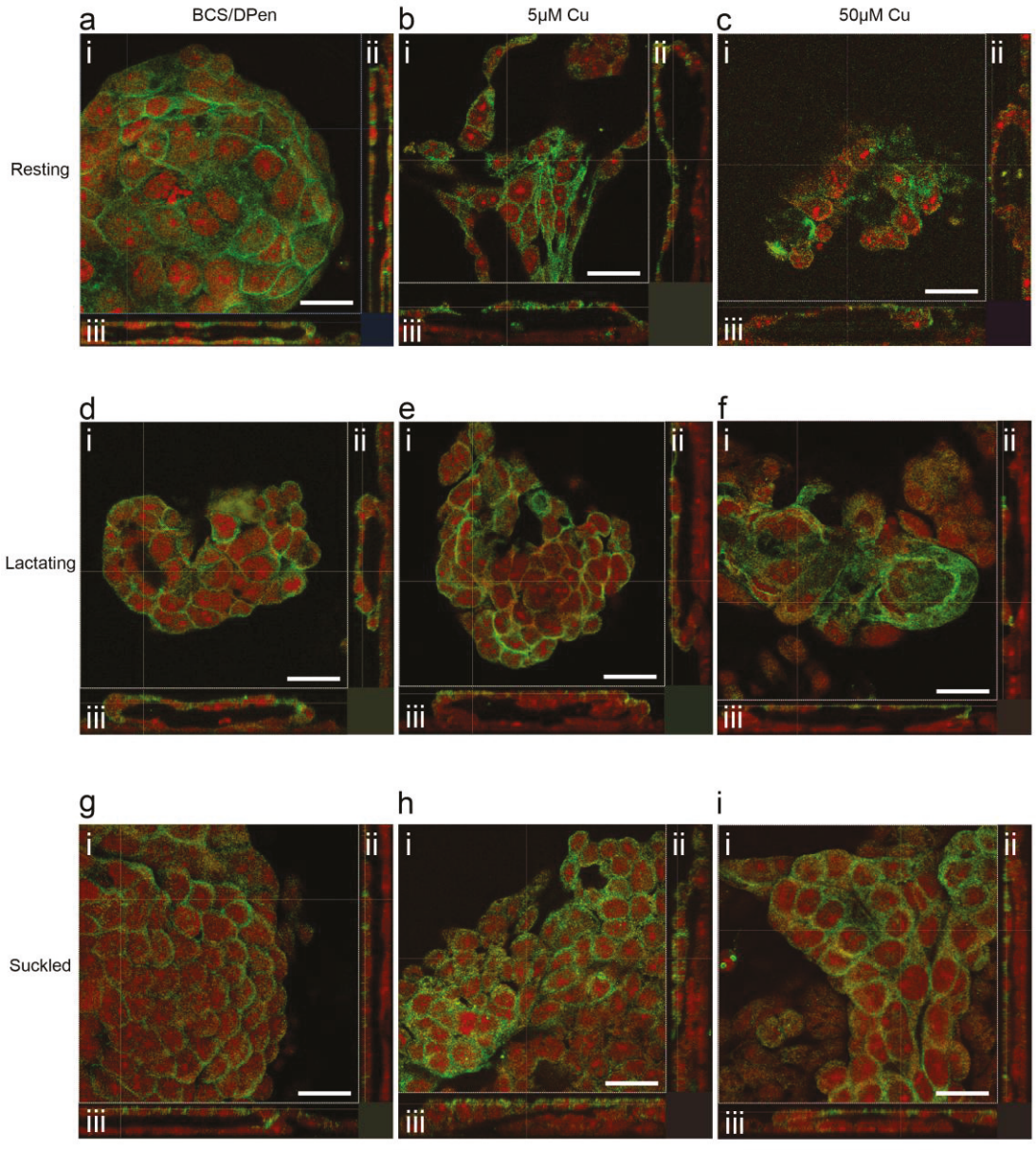
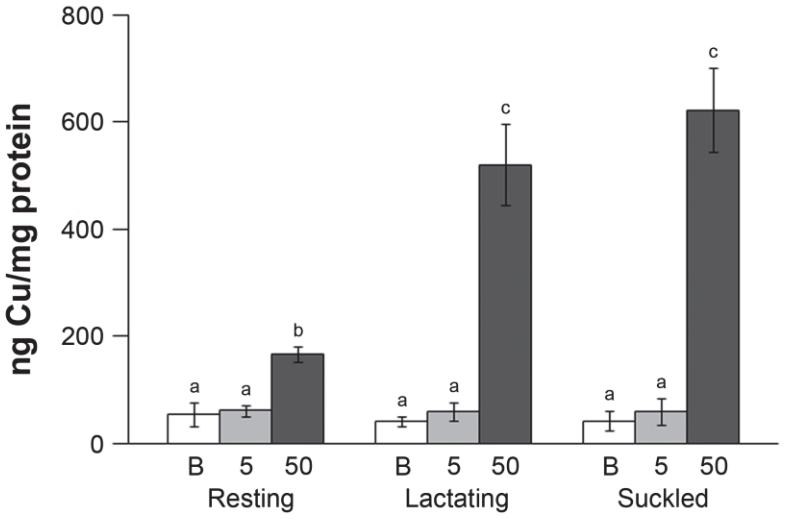

a
BCS/DPen
b
5μM Cu
c
50μM Cu
Resting
d
e
f
Lactating
g
h
i
Suckled
i
ii
iii

Figure 3.5 The intracellular copper levels of differentiated PMC42-LA cells treated with copper and lactational hormones

AAS analysis of copper levels within the PMC42-LA cells in response to BCS/DPen, 5 μM copper and 50 μM copper treatments for each of the resting, lactating and suckled models. Statistical variation is indicated by different letters above graph bars ($p<0.05$).


ng Cu/mg protein
800
600
400
200
0
a
a
b
a
a
c
a
a
c
B
5
50
Resting
Lactating
Suckled

3.2.8 The level and localisation of copper within PMC42-LA organoids was affected by copper and lactational hormone treatment

XFM analysis of PMC42-LA organoids revealed that the organoids accumulated copper more than the surrounding monolayer of cells. This was particularly evident in the lactating (Fig 3.6e and f) and suckled models (Fig 3.6h and i) treated with 5 μM and 50 μM copper, however less obvious in the resting model (Fig 3.6b and c). Copper was not detected in any of the BCS/DPen samples, similar to the undifferentiated cells (Fig 3.6a, d and g). Each copper distribution image is accompanied by a corresponding total fluorescence image showing the cells of the monolayer and the structure of the organoid.

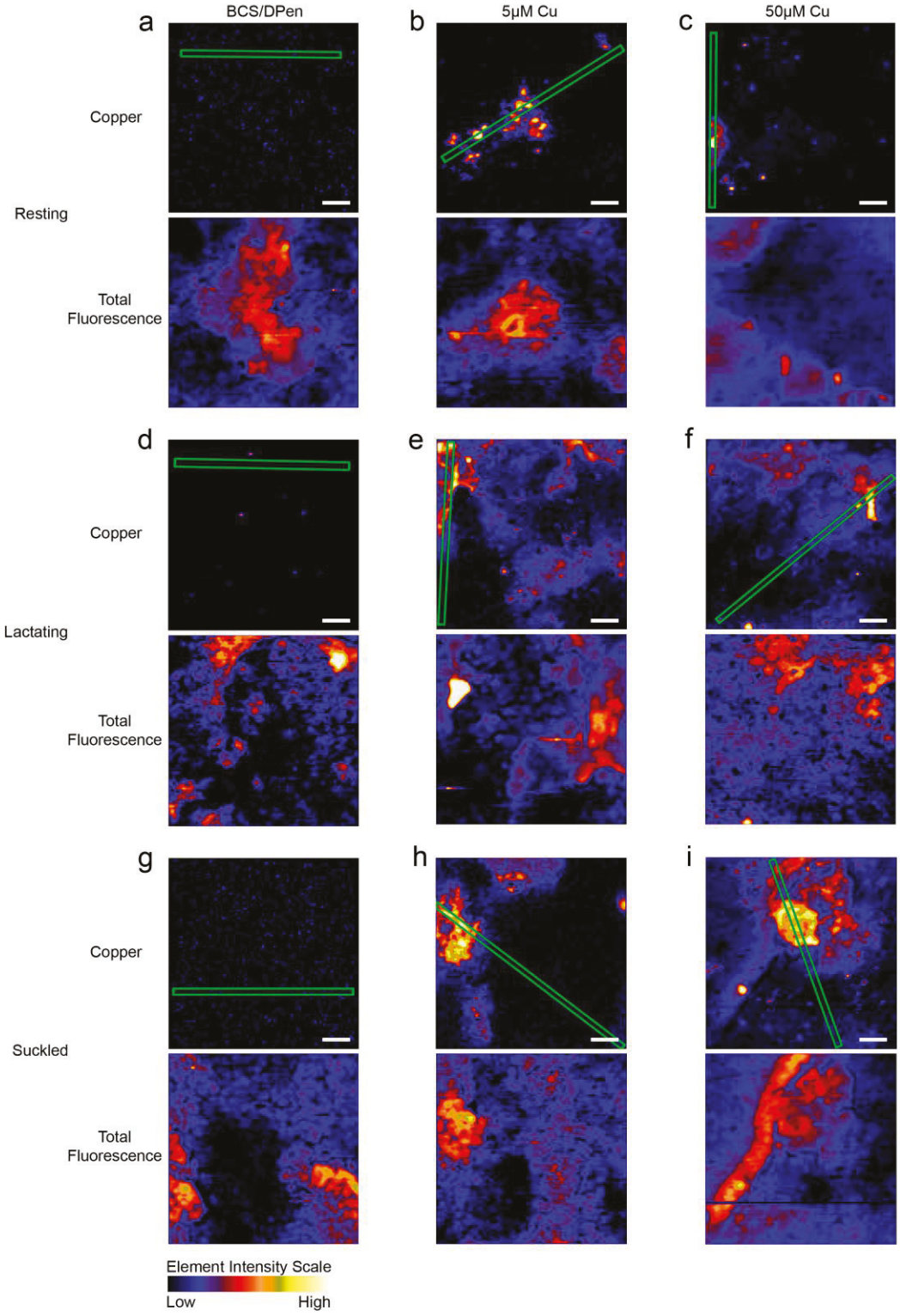
The graphs in figures 3.6j-r represent the levels of copper in the traverse regions defined by the green boxes shown on the images of figures 3.6a-i. There was a distinct correlation between copper levels and organoid localisation. The graphs of the 5 μM copper treated samples (Fig 3.6k,n and q) indicated low levels of copper (<5 ppm) in the monolayer region of cells, whereas in the presence of an organoid, the maximum levels rose to around 15-35 ppm for all models. The trend was similar in the 50 μM treated samples with the monolayer of the resting model giving readings no higher than 5 ppm (Fig 3.6l), however the lactating and suckled models (Fig 3.6o and r) had slightly higher levels in the monolayer (between 10-20 ppm) than the resting model. This level of copper is similar to the undifferentiated PMC42-LA cells and is consistent with the AAS data which showed that the levels in the 50 μM copper treated cells of the lactating and suckled models were similar to those of the undifferentiated cells, whereas the levels in the

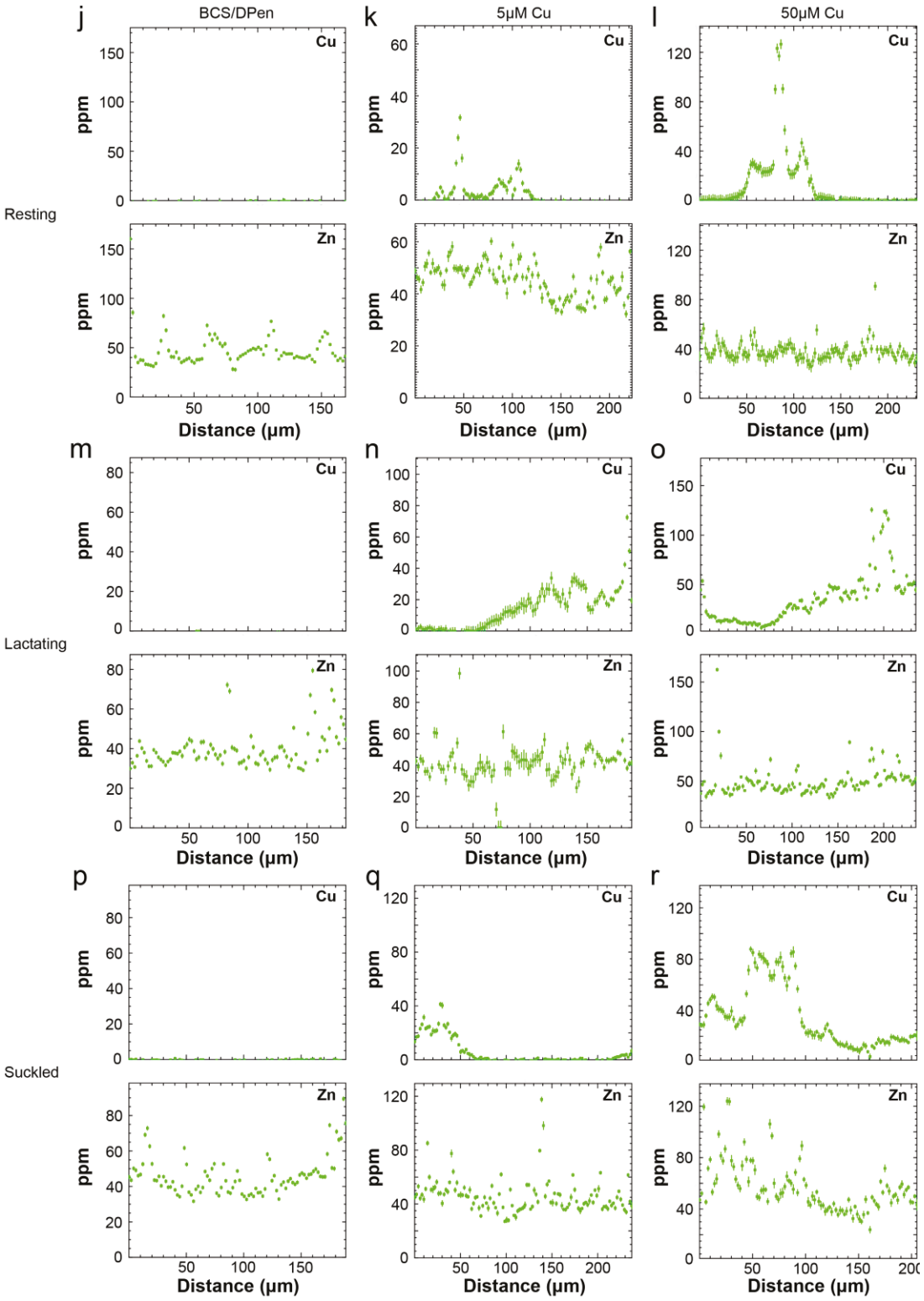
resting model were much lower. Like the 5 μM copper treated samples, levels rose significantly in the location of an organoid reaching 40 ppm in the resting model, 120 ppm in the lactating model and 85 ppm in the suckled model. The copper graphs of the BCS/DPen treated samples barely peaked above 0 ppm (Fig 3.6j, m and p).

To determine whether the accumulation within the organoid was copper specific, other elements were examined. Zinc showed little variation between monolayer and organoid giving readings of around 40-50 ppm. Similarly, iron was present in approximately 140-150 ppm for organoid and monolayer cells (data not shown). While there appears to be some regions of elevated zinc in the traverse region graphs (Fig 3.6j-r), these increases correspond to “hot spots” on the images and did not follow a trend consistent with accumulation specifically within an organoid or within the monolayer region.

Figure 3.6 Copper levels and localisation within differentiated PMC42-LA organoids

X-ray fluorescence images show the location of copper within the BCS/DPen (a, d and g), 5 μM copper (b, e and h) and 50 μM copper (c, f and i) treated cells in each of the resting (a, b and c), lactating (d, e and f) and suckled models (g, h and i). Total X-ray fluorescence images accompany the XFM copper images to show the position of each of the organoids. The colour scale bar shows the relative levels of copper from lowest (black) to highest (white). Length scale bar = 30 μm. The green line on each XFM image indicates the position of the traverse section displayed in figures 3.6 j-r. Metal levels within this traverse line are plotted on line graphs. Traverse sections through the organoids reveal the change in copper levels between the monolayers of cells and organoidal structures found in differentiated PMC42-LA cells. For each cell model treated with BCS/Dpen (j, m and p), 5 μM copper (k, n and q) and 50 μM copper (l, o and r), the graphs show the levels of copper and zinc, through each point of the traverse line shown in the copper images of figures 3.6 a-i. The x-axis shows distance along the traverse line in micrometers and the y-axis shows the levels of each element in ppm.


a
BCS/DPen
b
5μM Cu
c
50μM Cu
Copper
Resting
Total
Fluorescence
d
e
f
Copper
Lactating
Total
Fluorescence
g
h
i
Copper
Suckled
Total
Fluorescence
Element Intensity Scale
Low
High


j
BCS/DPen
k
5μM Cu
l
50μM Cu
Resting
m
n
o
Lactating
p
q
r
Suckled
Cu
Zn
ppm
Distance (μm)

3.3 Discussion

Copper is an essential component of milk, yet little is known about how this element is taken up by the mammary epithelial cells. There have been few reports on the regulation of CTR1 in the mammary gland in response to lactation. This research utilises previously developed cell culture models of the resting and lactating mammary gland in conjunction with a newly created suckled model to gain insight into the regulation of CTR1 and its involvement in the delivery of copper into the milk during the periods of lactation and suckling when copper and hormone levels fluctuate dramatically. As CTR1 is postulated to mediate copper absorption from the maternal bloodstream into the mammary epithelium for its incorporation into the milk and subsequent secretion, this work focuses on analysis of CTR1 protein expression in mammary epithelial cells in different lactational states.

Synchrotron X-ray fluorescence data revealed that the organoidal structures, which have been previously shown to contain mammary gland specific features including lipid granules, swollen endoplasmic reticulum and large secretory vacuoles (Ackland et al., 2001), accumulated copper, and this accumulation increased with the addition of lactational hormones in the representative lactating and suckled models. A 5-25 fold increase in copper was detected when comparing the highest levels in the central regions of the organoids to those of the surrounding monolayers. This increase is not due to the difference in thickness between the organoid and monolayer of cells. Organoids contain either two or three layers of cells and should not result in an increase greater than 2-3 fold depending on the organoid's thickness, or

possibly no detectable change at all, as was shown by the zinc and iron analysis. The copper localisation data suggest that the accumulation in the organoids is not an accumulation of copper within the cells, rather an accumulation of secreted copper within the lumen of the organoid. The AAS data indicated that there was no difference in copper levels between the cells treated with BCS/DPen and 5 μM copper for any of the differentiated models. In contrast, the XFM data showed the 5 μM copper treated organoid to be loaded with copper, whereas the BCS/DPen treated organoids had no observable copper. The monolayer areas of the BCS/DPen and 5 μM copper treated cultures had undetectable levels or in some cases barely detectable levels of copper; similar to the undifferentiated cells which also showed no difference in copper accumulation when analysed by AAS. Thus, the copper detected in the organoid is not accumulating in the cells, but concentrated within the lumen of the organoid. This copper would not be detected by AAS as it would be washed away during sample preparation in contrast to XFM sample preparation which maintains the organoid structures. The reason no copper was detected in the lumen of the BCS/DPen organoids, despite the fact that AAS analysis determined the levels to be equivalent between the BCS/DPen and 5 μM copper treated cultures, may be that the BCS/DPen samples would have a limited capacity to deliver copper into the lumen of the organoid as the majority of copper would be bound to the chelators and not biologically available for proteins to transport. The 50 μM copper treated cultures had more copper in the lumen and also in the cell monolayers than the 5 μM copper and BCS/DPen treated samples. This trend was reflected in the undifferentiated cells. The addition of lactational hormones to the 50 μM copper treated cultures increased cellular copper accumulation. Hormonally

treated samples also appear to have more copper in the lumen of the organoid compared to the resting model, while there was no significant difference between the lactating and suckled models. This indicates that the hormones are inducing the secretion of copper into the lumen of the organoids and similarly may mediate the movement of copper into the milk of a lactating mammary gland. This is consistent with the physiological observations described in rodents during lactation where copper is preferentially directed towards the mammary gland, presumably to be secreted into the milk to feed a suckling pup (Donley et al., 2002). The organoid data is also consistent with physiological observations that show copper levels in the milk alter in conjunction with circulating hormone levels during lactation. It was shown that when prolactin levels are elevated during early lactation, copper levels are high in the milk and gradually decrease as lactation progresses and circulating prolactin levels reduce (Kelleher and Lonnerdal, 2006).

Metallothionein 1 and 2 (MT) levels strongly correlated with the copper levels within the cells, being highest in cells containing high levels of copper in both the differentiated and undifferentiated cells. This is consistent with previous research that shows MT is regulated by copper and is elevated in conjunction with copper levels (Armendariz et al., 2004, Coyle et al., 2002, Prohaska and Gybina, 2004, Urani et al., 2003). It is also consistent with MT's role as a metallochaperone which sequesters excess metal ions like copper, zinc and cadmium within cells (Mehta et al., 2006, Sutherland and Stillman, 2011, Urani et al., 2003). The elevation of MT following hormone treatment is likely

a result of the additional copper found within the hormonally treated cells rather than a direct influence of the hormones themselves.

Using differentiated culture models it was shown that lactational hormones increased the total, as well as the plasma membrane bound CTR1 protein levels. It was evident in cells exposed to 50 μM copper, similar to the levels found in the lactating mammary gland, that the increased CTR1 protein caused by lactational hormones correlated with increased intracellular copper levels. The levels of CTR1 protein increased further in the suckled model compared to the lactating model suggesting that prolactin plays a role in CTR1 regulation. It has been shown previously that the monomeric form of CTR1 is responsive to hormones, with reduction in the Jeg-3 placental cell line upon exposure to progesterone, estrogen and insulin (Hardman et al., 2006). While this response appears to contradict the PMC42-LA cell culture data, it can be explained by understanding the copper fluxes in placenta (Hardman et al., 2006). In the mammary gland, copper moves from the maternal bloodstream towards the milk facilitated by CTR1 protein present at the basolateral membrane. In the placenta, the main movement of copper is from the maternal bloodstream to the foetal bloodstream, however in this instance, CTR1 is on the foetal side of the placenta (Hardman et al., 2006). Reduced CTR1 levels due to lactational hormones, as demonstrated in Jeg-3 cells, would decrease the amount of copper leaving the foetal bloodstream helping to drive the copper flow towards the growing foetus. CTR1 may have a protective role in this instance, removing excess copper from the foetal circulation back to the maternal bloodstream.

The *CTR1* mRNA results showed no changes in response to hormones or depleted or elevated copper levels. There have been few reports on the mRNA levels of *CTR1* in cultured cells, however experiments on differentiated intestinal Caco2 cells showed no response to changing copper levels (Bauerly et al., 2004, Tennant et al., 2002), while work with yeast and a small cell lung carcinoma cell line has shown that *CTR1* mRNA expression is influenced by changing copper levels (Kirchman and Botta, 2007, Song et al., 2008). In the latter research, total and membrane protein levels in these cells were not analysed. If the mechanisms controlling copper influx by CTR1 are cell type specific as suggested by Molloy and Kaplan, (2009), then perhaps it is possible that some cells regulate CTR1 by controlling transcription. In agreement with our findings, mice studies have shown that changes in circulating prolactin and copper concentration have no impact on the levels of *CTR1* mRNA in mice mammary glands (Gambling et al., 2004, Kelleher and Lonnerdal, 2006), also dietary copper intake has shown to have no impact on the *CTR1* mRNA levels in rat pups' small intestines (Bauerly et al., 2005).

The research presented in this chapter suggests that the changes in protein levels in response to copper and hormones may be a result of post translational CTR1 degradation as the transcription levels of this transporter was unaltered in any of the cell treatments. Degradation of endogenous CTR1 protein in response to copper has been reported a number of times (Holzer and Howell, 2006, Holzer et al., 2004a, Safaei et al., 2009) while it has also been shown that exogenously expressed, myc or GFP tagged CTR1 protein follows the same trend (Guo et al., 2004a, Guo et al., 2004b, Liu et

al., 2007, Petris et al., 2003). CTR1 is important for copper uptake and is possibly the primary copper influx protein in human cells (Eisses et al., 2005, Lee et al., 2002b), therefore down-regulating the levels of CTR1 in response to elevated copper may be essential to prevent cellular copper toxicity. This is clearly demonstrated by the results showing that the total CTR1 protein levels were reduced along with the levels of CTR1 protein at the plasma membrane. The addition of hormones seems to impede this degradation process, leading to the observed increase in CTR1 protein. Reduced degradation would enable more CTR1 to be retained at the membrane allowing more copper to enter the cells for transfer into the milk. CTR1 degradation has been reported previously in MDCK, HeLa and HepG2 cells in response to copper. HEK293 cells also showed a reduction in CTR1 protein at the plasma membrane, however this was not due to degradation of total CTR1 protein, rather a result of the internalisation of the protein complex into the cells by endocytosis (Molloy and Kaplan, 2009). Contrary to this T47D cells did not show a reduction in protein levels at the membrane in response to copper suggesting that the mechanism involved in controlling influx via CTR1 may be cell type specific (Molloy and Kaplan, 2009). Internalisation of CTR1 has been reported a number of times (Guo et al., 2004a, Guo et al., 2004b, Holzer and Howell, 2006, Holzer et al., 2004a, Liu et al., 2007, Petris et al., 2003) and is a logical process in the prevention of copper uptake into cells. CTR1 protein analysis data obtained from whole cell lysates and biotinylated plasma membrane fractions suggest that there may be some internalisation of CTR1 in addition to its degradation. The observation was made that the difference between the levels of CTR1 at the membrane in the BCS/DPen and 5 μM copper treated cells was far greater

than the difference between the total CTR1 levels of the same treatments. This was observed in undifferentiated cultures as well as the differentiated models. The same trend was also observed between the 5 μM copper and 50 μM copper treatments of the undifferentiated cells. These data indicate that when cells are depleted of copper, there is a higher proportion of the total CTR1 protein present at the membrane which is available for copper uptake. In the presence of copper, CTR1 protein is degraded, but internalisation also takes place.

Immunolocalisation studies revealed a membrane and cytoplasmic/vesicular pattern of CTR1 labelling. This is consistent with previous observations where researchers have shown that CTR1 predominantly localises at the plasma membrane (Eisses and Kaplan, 2002, Hardman et al., 2006, Holzer et al., 2004b, Kelleher and Lonnerdal, 2006, Klomp et al., 2003, Klomp et al., 2002, Lee et al., 2002a, Maryon et al., 2007, Petris et al., 2003) with some groups also reporting perinuclear (Eisses and Kaplan, 2002, Klomp et al., 2002) and cytoplasmic/vesicular (Guo et al., 2004b, Holzer et al., 2004a, Kelleher and Lonnerdal, 2006, Klomp et al., 2002, Safaei et al., 2009) distribution, however the localisation observed between cells lines seems to vary widely (Klomp et al., 2002). While the biotinylation and whole cell lysate data suggest some internalisation of CTR1 protein from the membrane, immunofluorescent staining of CTR1 did not reveal differences following treatment with copper and hormones, a trend also observed in Jeg-3, HeLa, Caco2, HEK293 cells (Eisses et al., 2005, Hardman et al., 2006, Klomp et al., 2002) and in mammary gland tissues between early and late lactation when copper and hormone levels differ greatly (Kelleher and Lonnerdal, 2006).

Several studies have reported internalisation of CTR1 from the membrane following copper treatment (Guo et al., 2004a, Guo et al., 2004b, Liu et al., 2007, Molloy and Kaplan, 2009) by clathrin dependent macropinocytosis (Holzer and Howell, 2006, Petris et al., 2003) which preceeds proteosomal degradation (Holzer and Howell, 2006, Jandial et al., 2009, Liu et al., 2007). CTR1 internalises from the membrane into cytoplasmic vesicles (Guo et al., 2004a) in which it has been shown to co-localise with transferrin receptor in endosomes (Bauerly et al., 2004, Eisses et al., 2005, Kelleher and Lonnerdal, 2006, Petris et al., 2003). Such slight movements from plasma membrane to endosomal compartments upon treatment with copper would be indistinguishable using single labelled fluorescence microscopy.

X-Z sections produced by confocal microscopy revealed that CTR1 localised towards the basolateral side of the cells in PMC42-LA organoid structures. Studies on polarised MDCK, OK and Caco2 cultured cells have identified CTR1 at the basolateral membrane using biotinylation (Molloy and Kaplan, 2009, Zimnicka et al., 2007). This is consistent with mouse tissues that have shown CTR1 to be basolaterally localised in the mammary gland (Kelleher and Lonnerdal, 2003, Kelleher and Lonnerdal, 2006, Llanos et al., 2008) and placenta (Hardman et al., 2006).

In the lactating cells treated with 50 μM copper, there was a higher level of copper in the cells compared to the resting model. CTR1 may be mediating additional copper uptake that is not balanced by efflux from the cells resulting in an increase in intracellular copper, reminiscent of the elevated copper levels observed in lactating mammary epithelial cells. In the suckled model a

further up-regulation of CTR1 levels was observedl. It is possible that prolactin has also facilitated greater efflux of copper from the apical membrane of the epithelial cells.

It is proposed that the increase in CTR1 induced by exposure to hormones increases the flux of copper through the cell into the milk. This may be in conjunction with ATP7B, reported to be a key protein involved in delivery of copper into milk (Michalczyk et al., 2000, Rauch, 1983). ATP7B has been shown to traffic towards the lumen of the organoids in response to hormones in the same PMC42-LA mammary culture models (Michalczyk et al., 2008). This would assist the copper taken up by CTR1 to be moved across the apical membrane.

Suckling and hyperprolactinemia stimulated copper transport into the milk of rodents by moving more CTR1 protein to the basolateral membrane (Kelleher and Lonnerdal, 2006). This, along with the data presented in this chapter, suggest that the elevation of prolactin causes an increase in copper transport through the mammary epithelial cells.

Cultured HC11 mammary epithelial cells showed no change in the levels of CTR1 protein in response to 1 μg/mL prolactin (Kelleher and Lonnerdal, 2006), however this could be a result of the cells being undifferentiated. It has been shown that copper transporting proteins, like ATP7A, can be unresponsive to hormones in undifferentiated cells (Ackland et al., 1997), while being influenced in differentiated cells (chapter 6) and tissues (Ackland et al., 1999).

3.4 Conclusion

The development of a differentiated mammary gland model (PMC42-LA) that mimics the human breast epithelium during lactation and periods of suckling, has enabled the investigation of copper and hormonal regulation of CTR1 and has provided evidence that CTR1 plays a role in copper homeostasis in breast epithelial cells. This work on the cell culture models representing three different physiological states has shown that hormones, specifically prolactin, influence the total and membrane bound CTR1 protein levels. This is associated with an increase in intracellular copper levels. Synchrotron X-ray fluorescence analysis revealed that copper is secreted into the lumen of the organoids as it is in the alveolar lumen of the mammary gland; an effect that was enhanced by the addition of lactational hormones. It is proposed that while extracellular copper concentrations regulate the levels of CTR1 through degradation, the addition of lactational hormones, most likely prolactin, initiates mechanisms that inhibit the degradation of CTR1 allowing greater uptake of copper into the cells thus enabling more copper to be secreted into milk to feed and nourish the suckling infant.

CHAPTER 4

The expression and secretion of ceruloplasmin protein is influenced by copper and lactational hormones in PMC42-LA human breast cells

4.1 Introduction

The study presented in chapter 3 focused on the uptake of copper into the mammary epithelial cells by CTR1. This shed light on the processes involved in copper uptake as a means of regulating the delivery of copper into milk to nourish a suckling neonate. While it is important to understand how copper uptake into the mammary epithelial cells is regulated through CTR1, it is also important to understand the processes involved in directly delivering copper into the secreted milk.

Ceruloplasmin (Cp) has roles in copper transport and iron oxidation. There are two forms of Cp that have been identified; a secreted (sCp) and a membrane bound glycosylphosphatidylinositol-linked (GPI-Cp) form (Hellman et al., 2002b, Kono et al., 2006b, Mittal et al., 2003, Patel et al., 2000, Platonova et al., 2007, Terada et al., 1995). Each of these forms exist in two states; copper bound feroxidase active holo-Cp and copper depleted ferroxidase inactive apo-Cp (Hellman et al., 2002a, Hochstrasser et al., 2005, Kim et al., 2009, Kono et al., 2006a, Kono et al., 2010, Sato and Gitlin, 1991, Terada et al., 1995, Zaitseva et al., 1996). Cp that is secreted from mammary epithelial cells binds up to 85% of copper in milk (Platonova et al., 2007, Tsymbalenko et al., 2009), thus Cp-bound copper is a significant source of copper for the neonate. Cp's ferroxidase activity enables it to oxidise Fe (II) to the more inert Fe (III) form. It is likely that Cp helps load iron onto lactoferrin, which carries iron in milk (Dorea, 2000, González-Chávez et al., 2009, Ha-Duong et al., 2010, Kawakami et al., 1990, Sabatucci et al., 2007, Sokolov et al., 2006, Zakharova et al., 2000). This suggests Cp also has an

important role in the homeostasis of iron. The membrane bound GPI- Cp is thought to oxidise iron and facilitate iron efflux (Chang et al., 2007, Jeong and David, 2003, Kono et al., 2006a).

The differentiated culture models introduced in chapter 3 that represent the resting, lactating and suckled mammary epithelium have been used throughout chapter 4 to analyse the forms of Cp found in mammary epithelial cells. The impact of copper levels and lactational hormones on the expression of the ferroxidase active holo-Cp and its secretion from the cells has been investigated. The presence of the membrane bound GPI-Cp as well as a direct interaction between ATP7B and Cp was also investigated in this chapter. These studies contribute to the elucidation of the role of Cp in mammary epithelial cells, further enhancing the understanding of the mechanisms controlling copper delivery into milk.

4.2 Results

4.2.1 Two forms of *Cp* transcripts were present in PMC42-LA cells

RT-PCR was performed to identify the transcripts of two alternatively spiced forms of *Cp*, secreted (*sCp*) and glycosylphosphatidylinositol-linked (*GPI-Cp*), in PMC42-LA cells. Using previously published primer sequences (Banha et al., 2008), bands of 157 bp (*sCp*) and 407 bp (*GPI-Cp*) were identified, which corresponded to the expected size of the PCR products (Fig 4.1). The lower band in the GPI-Cp lane is an unidentified artifact which appears in the original method (Banha et al., 2008).

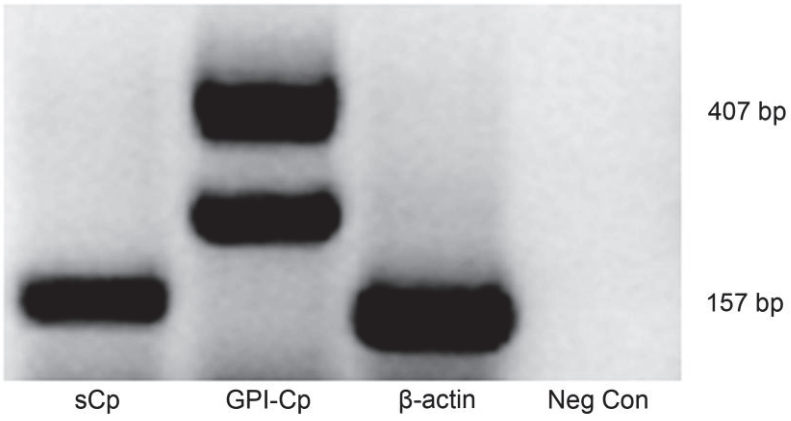
4.2.2 Copper affected apo and holo-Cp protein levels, but not localisation or mRNA levels in undifferentiated PMC42-LA cells

To determine the effect of copper on total *Cp* mRNA, Quantitative Real Time PCR was performed on undifferentiated PMC42-LA cells. The data showed no change in the levels of total *Cp* mRNA in response to BCS/DPen (102.5 ± 8.7%), 5 μM copper (100.0 ± 16.1%) and 50 μM copper (81.6 ± 6.4%) treatment ($p>0.05$) (Fig 4.2a).

Western blot analysis of Cp protein revealed bands of 132 kDa, 130 kDa and 95 kDa corresponding to the non-copper bound apo-ceruloplasmin (apo-Cp), unglycosylated ceruloplasmin and the copper bound holo-ceruloplasmin (holo-Cp) forms respectively (Fig 4.2b). Densitometric analysis demonstrated that apo-Cp protein levels declined with increasing copper concentrations (BCS/DPen – 153.5 ± 42.3%, 5 μM copper – 100.0 ± 19.0%, 50 μM copper – 89.1 ± 19.4%) and the difference between the BCS/DPen and 50 μM copper

Figure 4.1 Detection of *sCp* and *GPI-Cp* mRNA in PMC42-LA cells by RT-PCR

This 1.5% agarose gel shows the PCR products of *sCp* (lane 1), *GPI-Cp* (lane 2 – top band) and the *β-actin* positive control (lane 3). The no cDNA negative control with no PCR product (lane 4).


407 bp
157 bp
sCp
GPI-Cp
β-actin
Neg Con

treated cells was statistically significant ($p<0.05$). Holo-Cp levels increased significantly in the presence of 50 μM copper compared with the copper depleted BCS/DPen treated cells ($p<0.05$), however no significant difference was observed between these two treatments and the 5 μM copper basal control treatment (BCS/DPen – 68.1 ± 21.1%, 5 μM copper – 100.0 ± 20.6%, 50 μM copper – 113.0 ± 14.8%). The band at 130 kDa has previously been identified as an unglycosylated form of Cp (Boivin et al., 2001).

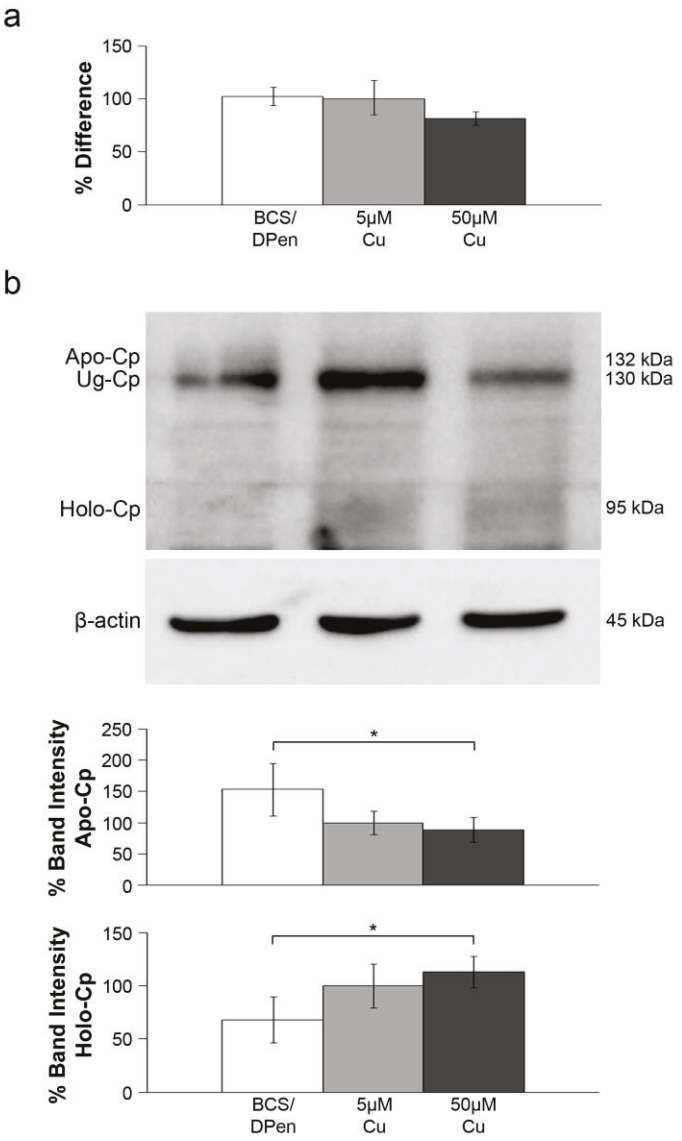
Immunofluorescence confocal microscopy was used to identify the distribution of Cp in PMC42-LA cells treated with BCS/DPen (i), 5μM copper (ii) or 50μM copper (iii). Cp was distributed throughout the cytoplasm, however no differences were observed between treatments (Fig 4.2c).

4.2.3 Copper treatment influenced the levels of secreted apo and holo-Cp, but not total GPI-Cp levels

To identify whether Cp was secreted from PMC42-LA cells, culture medium was collected from cells grown in BCS/DPen, 5 μM copper and 50 μM copper for 3 days. Western blotting analysis of culture medium showed bands of 132 kDa and 95 kDa corresponding to the apo-Cp and holo-Cp as in the lysates. The 130 kDa band was identified as the bovine Cp (Boivin et al., 2001, Patel and David, 1997) that is present in the fetal bovine serum of the RPMI culture medium and has no relevance to the human model (Fig 4.3a). Apo-Cp levels decreased in response to copper in a similar fashion to the apo-Cp in the whole cell lysates (BCS/DPen – 270.1 ± 78.7%, 5 μM copper –100.0 ± 37.7%, 50 μM copper – 93.8 ± 30.1%). There was no significant

Figure 4.2 Cp mRNA and protein levels in response to copper treatments in undifferentiated PMC42 cells

(a) Relative *Cp* mRNA levels of 50 μM copper and BCS/DPen treatments compared to that of the 5 μM copper treated cells. The bar graph shows the mean percentage of relative mRNA ±SD from three independent experiments normalised against *β-actin* obtained by qRT-PCR. (b) Representative Cp Western blot analysis of 60 μg of undifferentiated PMC42-LA cell extracts. Lane 1, BCS/DPen treatment; lane 2, 5 μM copper treatment; lane 3, 50 μM copper treatment. β-actin was used as a loading control. Desitometric analysis show the intensity of the 132 kDa apo-Cp and 95 kDa holo-Cp bands expressed as a mean percentage of arbitrary units ±SD compared to the 5 μM copper treated sample normalised against β-actin. The 130 kDa band is an unglycosylated form of Cp (Ug-Cp). This figure shows representative results of three independent experiments. Asterisk indicates significant difference between samples ($p<0.05$). (c) Immunolocalisation of Cp (green) in PMC42-LA cells treated with BCS/DPen (i), 5 μM copper (ii) and 50 μM copper (iii). Nuclei were stained with ethidium bromide (red). Scale bar = 10 μm.


a
% Difference
150
100
50
0
BCS/
DPen
5μM
Cu
50μM
Cu
b
Apo-Cp
Ug-Cp
Holo-Cp
β-actin
132 kDa
130 kDa
95 kDa
45 kDa
% Band Intensity
Apo-Cp
250
200
150
100
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% Band Intensity
Holo-Cp
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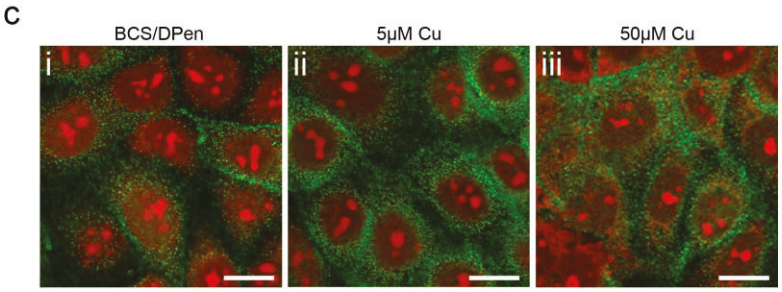
BCS/
DPen
5μM
Cu
50μM
Cu


c
BCS/DPen
5μM Cu
50μM Cu
i
ii
iii

difference between the 5 μM and 50 μM copper treatments, however in both of these treatments the cells secreted significantly less apo-Cp than the BCS/DPen treated cells ($p<0.05$). The 95 kDa secreted holo-Cp levels followed a similar trend to the whole cell lysates, increasing significantly in the presence of copper compared to the copper depleted BCS/DPen sample ($p<0.05$) (BCS/DPen – 46.2 ± 18.0%, 5 μM copper – 100.0 ± 14.7%, 50 μM copper – 109.2 ± 24.1%). Blood and milk samples were used as positive controls to demonstrate the presence of apo-Cp and holo-Cp in extracellular fluids.

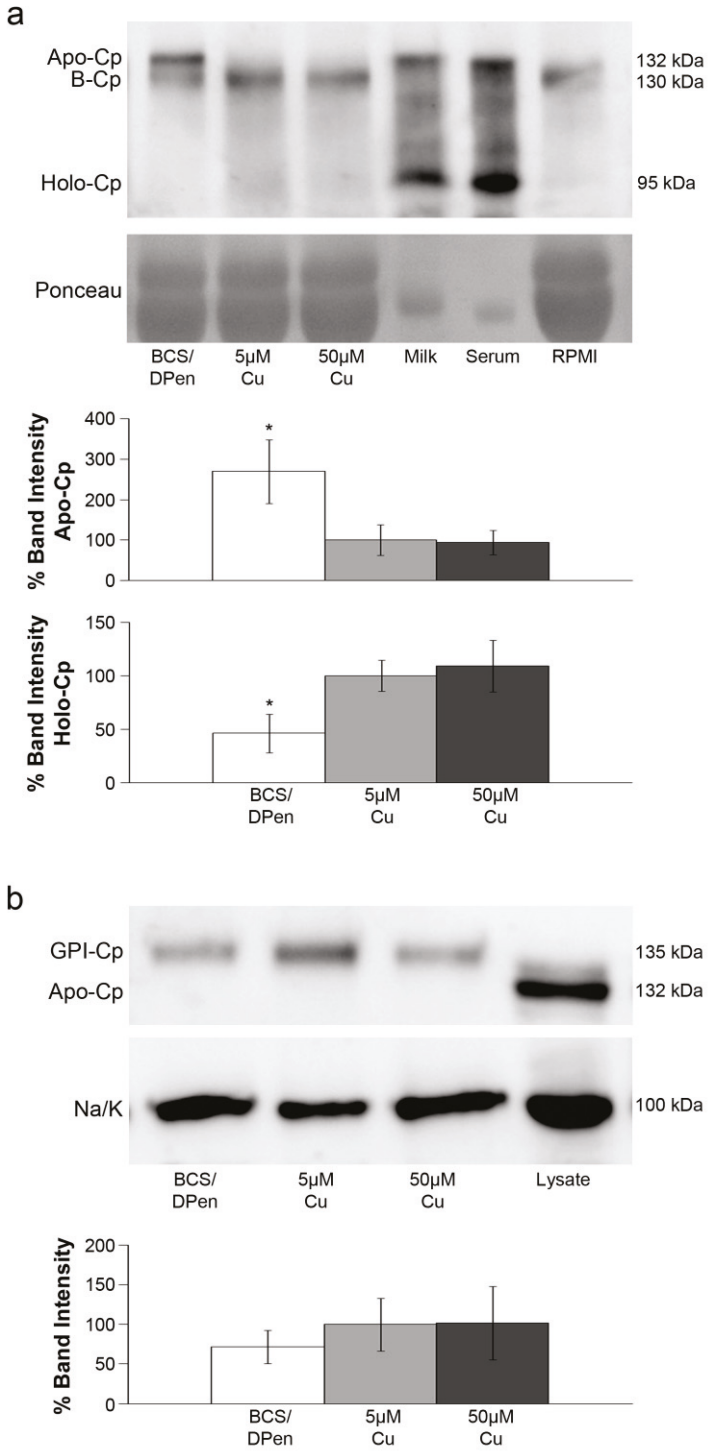
GPI-Cp was not detected in whole cell lysates possibly due to its lower abundance compared with sCp, however surface biotinylation enabled us to quantify the amount of plasma membrane bound GPI-Cp. The 135 kDa band detected in all samples corresponds to GPI-linked Cp, slightly larger than the 132 kDa apo-Cp band. (Fig 4.3b). Densitometric analysis revealed that copper treatment had no significant effects on the levels of plasma membrane bound GPI-Cp (BCS/DPen – 71.7 ± 21.3%, 5 μM copper – 100 ± 33.3%, 50 μM copper – 101.7 ± 46.2%) ($p>0.05$).

4.2.4 Copper levels influenced the proportion of apo and holo-Cp in differentiated PMC42-LA cells, while lactational hormones affected mRNA levels

The differentiated PMC42-LA culture models representative of the resting, lactating and suckled mammary glands described in chapter 3 have been used to investigate the effects of copper and lactational hormones upon Cp

Figure 4.3 Secreted Cp and GPI-Cp protein levels in response to copper in undifferentiated PMC42 cells

(a) Representative Cp Western blot of 60 µg of protein obtained from the growth medium collected at the conclusion of the experiment from cultured PMC42-LA cells treated with copper and copper chelators. Lane 1, BCS/DPen treatment; lane 2, 5 µM copper treatment; lane 3, 50 µM copper treatment. Ponceau stain was used as a loading control. Densitometric analysis show the intensity of the 132 kDa apo-Cp and 95 kDa holo-Cp bands expressed as a mean percentage of arbitrary units ±SD compared to the 5 µM copper treated sample normalised against Ponceau S stain. This figure shows representative results of three independent experiments. Asterisks indicate significant differences between samples ($p<0.05$). Milk (lane 4) and serum (lane 5) were used as positive controls for identification of secreted forms of Cp. RPMI growth medium (lane 6) was used to confirm that the 130 kDa band present in all samples is bovine Cp (B-Cp) from the culture medium. (b) The graph indicates densitometric values obtained from Western blot analysis of biotinylated GPI-Cp protein bands in response to BCS/DPen (lane 1), 5 µM copper (lane 2) and 50 µM copper (lane 3). Values are expressed as a mean percentage of arbitrary units ±SD compared to the 5 µM copper treated sample normalised against Na/K ATPase. Results are representative of three independent experiments. The BCS/DPen treated lysate (lane 4) shows the band size of apo-Cp in comparison to GPI-Cp.


a
Apo-Cp
B-Cp
Holo-Cp
132 kDa
130 kDa
95 kDa
Ponceau
BCS/ DPen
5μM Cu
50μM Cu
Milk
Serum
RPMI
% Band Intensity Apo-Cp
400
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200
100
0
*
% Band Intensity Holo-Cp
150
100
50
0
*
BCS/ DPen
5μM Cu
50μM Cu
b
GPI-Cp
Apo-Cp
135 kDa
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Na/K
100 kDa
BCS/ DPen
5μM Cu
50μM Cu
Lysate
% Band Intensity
200
150
100
50
0
BCS/ DPen
5μM Cu
50μM Cu

to elucidate its role in delivering copper to milk. The elevated copper treatment corresponds to maternal serum copper levels in the early stages of lactation, while 5 μM copper treatment represents resting conditions. Quantitative Real Time PCR was used to analyse the total *Cp* mRNA levels in response to copper in each of the three differentiated PMC42-LA models. No significant differences were observed between the various copper treatments of the resting model (BCS/DPen – 96.8 ± 27.6%, 5 μM copper - 100 ± 10.7%, 50 μM copper – 113.7 ± 10.3%), lactating model (BCS/DPen – 199.6 ± 49.0%, 5 μM copper – 240.6 ± 35.4%, 50 μM copper – 216.7 ± 64.2%) or the suckled model (BCS/DPen – 203.6 ± 46.5%, 5 μM copper – 191.1 ± 42.2%, 50 μM copper – 252.7 ± 42.1%) ($p>0.05$). However hormones significantly increased *Cp* mRNA levels in the lactating and suckled model compared to the resting model for all of the copper treatments ($p<0.05$). No significant difference was observed in any of the treatments between the lactating and suckled models ($p>0.05$) (Fig 4.4a).

Total Cp protein levels were also analysed in response to copper and hormone treatments (Fig 4.4b). As it was shown in the undifferentiated cells, apo-Cp decreased in response to copper in the resting model (BCS/DPen – 234.3 ± 40.1%, 5 μM copper – 100.0 ± 17.7%, 50 μM copper – 107.0 ± 21.9%), lactating model (BCS/DPen – 277.4 ± 63.6%, 5 μM copper – 132.0 ± 21.0%, 50 μM copper – 123.9 ± 35.5%) and suckled model (BCS/DPen – 295.7 ± 25.4%, 5 μM copper – 101.1 ± 45.0%, 50 μM copper – 105.4 ± 57.2%), while holo-Cp levels increased in the presence of copper; resting model (BCS/DPen – 46.0 ± 19.6%, 5 μM copper – 100.0 ± 22.5%, 50 μM copper – 112.9 ± 16.5%), lactating model (BCS/DPen – 68.7 ± 20.6%, 5 μM

copper – 151.8 ± 27.7%, 50 μM copper – 143.1 ± 20.6%), suckled model (BCS/DPen – 72.6 ± 25.7%, 5 μM copper – 126.1 ± 17.9%, 50 μM copper – 162.9 ± 44.0%). Significant variation was observed between bands of the BCS/DPen and copper treated samples for both apo and holo-Cp in each of the cell culture models ($p<0.05$), however no statistical difference was observed between the 5 μM and 50 μM copper treatements ($p>0.05$). The protein levels of both the apo-Cp and holo-Cp were unaffected by the presence of lactational hormones ($p>0.05$).

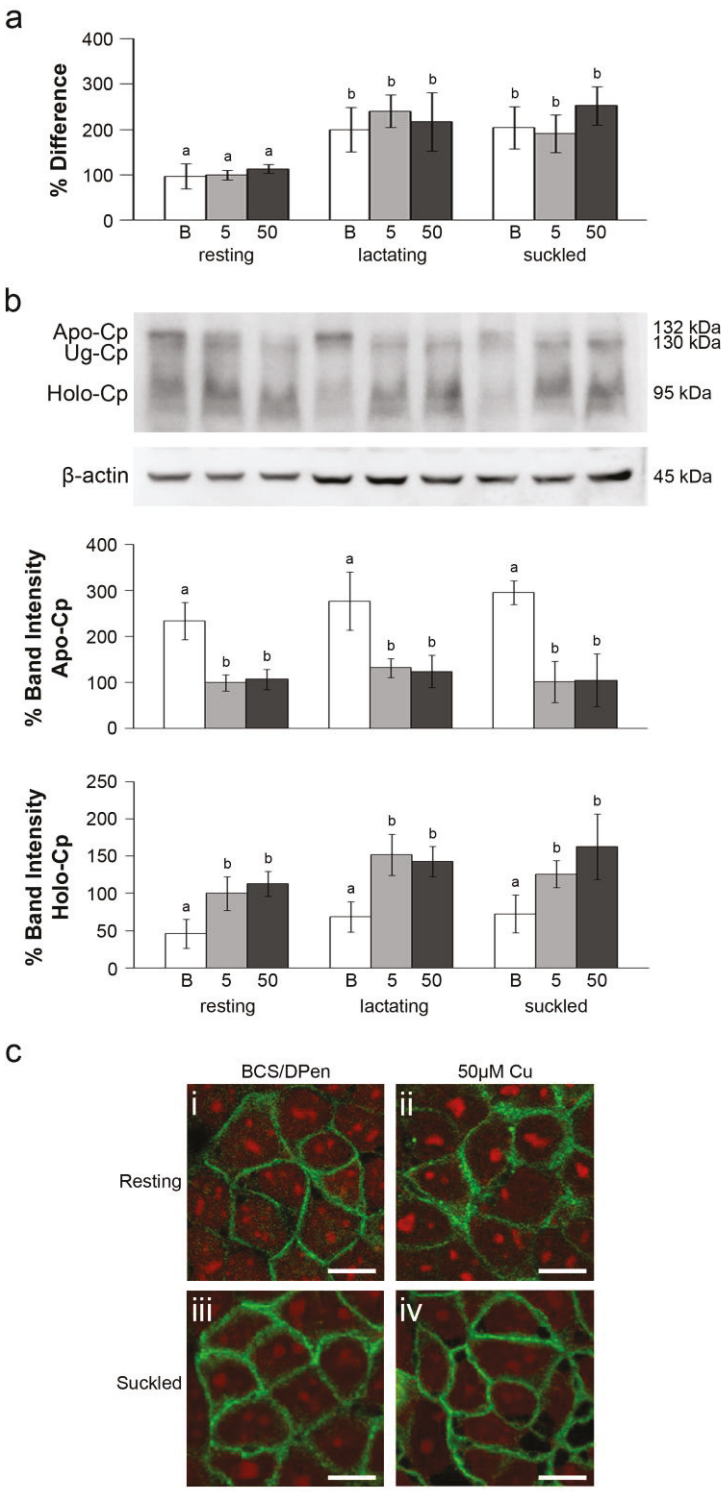
The distribution of Cp in differentiated PMC42-LA cells treated with BCS/DPen, 5 μM copper or 50 μM copper in each of the resting, lactating and suckled models, were analysed by immunofluorescent microscopy (Fig 4.4c). Cp was distributed throughout the cytoplasm and plasma membrane in all samples. Variation in copper levels or the addition of lactational hormones had no effect upon Cp localisation. The BCS/DPen and 50 μM copper treated resting and suckled models are shown here as examples of the distribution of Cp within the cells following treatments.

4.2.5 Copper and hormonal treatments influenced Cp secretions, but not GPI-Cp levels

Growth medium was collected from the differentiated cells grown on Transwell filters and analysed by Western blot (Fig 4.5a). Similar to the differentiated cell lysates, apo-Cp decreased in response to copper in the resting model (BCS/DPen – 176.6 ± 26.7%, 5 μM copper – 100.0 ± 11.4%, 50 μM copper – 95.8 ± 6.8%), lactating model (BCS/DPen – 515.2 ± 92.7%, 5 μM copper – 250.4 ± 48.7%, 50 μM copper – 254.0 ± 39.8%) and suckled

Figure 4.4 Cp mRNA levels and protein expression and localisation in differentiated PMC42-LA

(a) Relative mRNA levels of *Cp* in response to copper and hormones obtained by qRT-PCR. The bar graph indicates the mean percentage differences ±SD compared to the 5 μM copper treated cells of the resting model. *Cp* mRNA levels were normalised against *β-actin*. BCS/DPen = B, 5 μM copper = 5, 50 μM copper = 50. Statistical variation is indicated by different letters above graph bars ($p<0.05$). (b) Representative Western blots of 60μg of protein extract show Cp in the resting breast model (lanes 1-3), lactating model (lanes 4-6) and suckled model (lanes 7-9) treated with BCS/DPen (lanes 1, 4 and 7), 5 μM copper (lanes 2, 5 and 8) and 50 μM copper (lanes 3, 6 and 9). The β-actin blot demonstrates relative loading of samples. Desitometric analysis show the intensity of the 132 kDa apo-Cp and 95 kDa holo-Cp bands expressed as a mean percentage of arbitrary units ±SD compared to the 5 μM copper treated sample of the resting model normalised against β-actin. The 130 kDa band is an unglycosylated form of Cp (Ug-Cp). Results are representative of three independent experiments. Statistical variation is indicated by different letters above graph bars ($p<0.05$). (c) Immunofluorescence analysis of Cp (green) in differentiated PMC42-LA cells. Cp protein localisation in the resting (i and ii) and suckled (iii and iv) breast cell model in response to BCS/DPen (i and iii) and 50 μM copper (ii and iv). Nuclei were stained with ethidium bromide (red). Scale bar = 10 μm.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BCS/DPen
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Resting
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i
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iii
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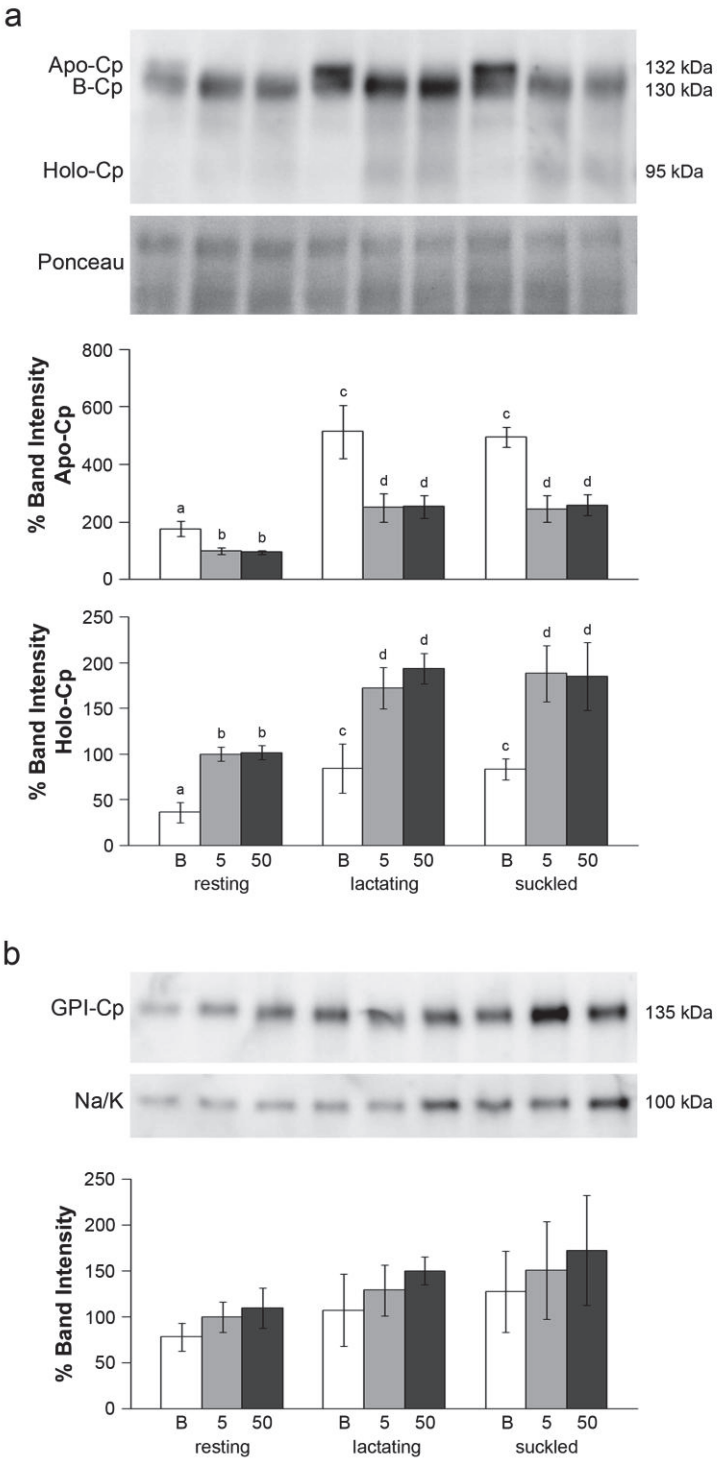
model (BCS/DPen – 496.2 ± 34.8%, 5 μM copper – 245.3 ± 46.6%, 50 μM copper – 258.4 ± 36.5%); the variation between the BCS/DPen samples and the copper treated samples was statistically significant ($p<0.05$). Holo-Cp expression was more prominent in the differentiated cultures and followed the same trend as the undifferentiated cells, showing a statistically significant increase in holo-Cp secretion in the presence of copper ($p<0.05$); resting model (BCS/DPen – 36.1 ± 10.9%, 5 μM copper – 100.0 ± 7.7%, 50 μM copper – 101.6 ± 7.8%), lactating model (BCS/DPen – 84.5 ± 26.6%, 5 μM copper – 172.6 ± 22.6%, 50 μM copper – 193.5 ± 16.7%), suckled model (BCS/DPen – 83.6 ± 11.6%, 5 μM copper – 188.5 ± 30.7%, 50 μM copper – 185.5 ± 37.3%). The addition of lactational hormones had a significant effect on the level of Cp secreted from cells. Both apo-Cp and holo-Cp were elevated in secretions from the suckled and lactating models compared to the resting model for all copper treatments ($p<0.05$), however no statistically significant difference was observed between the lactating and suckled models ($p>0.05$).

Membrane bound GPI-Cp was isolated from cells using surface biotinylation to identify variations in protein levels in response to copper or lactational hormones (Fig 4.5b). Data from the resting model (BCS/DPen – 78.3 ± 14.9%, 5 μM copper – 100.0 ± 16.2%, 50 μM copper – 109.9 ± 21.6%), lactating model (BCS/DPen – 107.6 ± 39.5%, 5 μM copper – 129.3 ± 27.7%, 50 μM copper – 150.0 ± 15.1%) and suckled model (BCS/DPen – 127.6 ± 44.0%, 5 μM copper – 150.6 ± 52.8%, 50 μM copper – 172.5 ± 59.5%) indicated that copper and hormone treatments had no significant influence on the expression of GPI-Cp ($p>0.05$).

Figure 4.5 Cp secretion and GPI-Cp expression in differentiated PMC42-LA cells treated with copper and lactational hormones

(a) Representative Western blot of 60µg of growth medium collected from cultured PMC42-LA cells treated with hormones, copper and copper chelators. The resting model (Lane 1-3), lactating model (Lane 4-6) and sucked model (Lane 7-9) were each treated with BCS/DPen (Lane 1, 4 and 7), 5 µM copper (Lane 2, 5 and 8) or 50 µM copper (Lanes 3, 6 and 9). Desitometric analysis shows the intensity of the 132 kDa apo-Cp and 95 kDa holo-Cp bands expressed as a mean percentage of arbitrary units ±SD compared to the 5 µM copper treated sample of the resting model normalised against Ponceau S stain. The 130 kDa band is the bovine Cp from the RPMI growth medium (B-Cp). Results are representative of three independent experiments. The statistical significance of results is indicated by different letters above graph bars ($p<0.05$). (b) Surface biotinylated GPI-Cp protein was analysed by Western blot. This representative Western blot of extracellularly biotinylated protein extract shows the GPI-Cp band in the resting breast model (lanes 1-3), lactating model (lanes 4-6) and suckled model (lanes 7-9) treated with BCS/DPen (lanes 1, 4 and 7), 5 µM copper (lanes 2, 5 and 8) and 50 µM copper (lanes 3, 6 and 9). Densitometric analysis shows mean intensity of the 135 kDa GPI-Cp bands expressed as a percentage of arbitrary units ±SD compared to the 5 µM copper treatment of the resting model. Densitometric analysis of GPI-Cp bands were normalised against the Na/K ATPase. Results are representative of three independent experiments.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Ponceau
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lactating
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b
GPI-Cp
Na/K
135 kDa
100 kDa
% Band Intensity
B 5 50
resting
lactating
suckled

4.2.6 Cp interacted with ATP7B, but not ATP7A in PMC42-LA cells

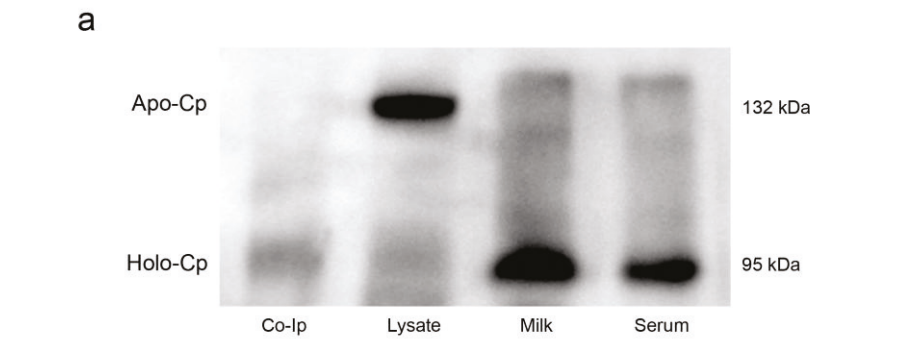
To detect whether ATP7A or ATP7B directly interact with Cp in PMC42-LA cells both co-immunoprecipitation (Co-Ip) and co-immunofluorescence microscopy experiments were performed.

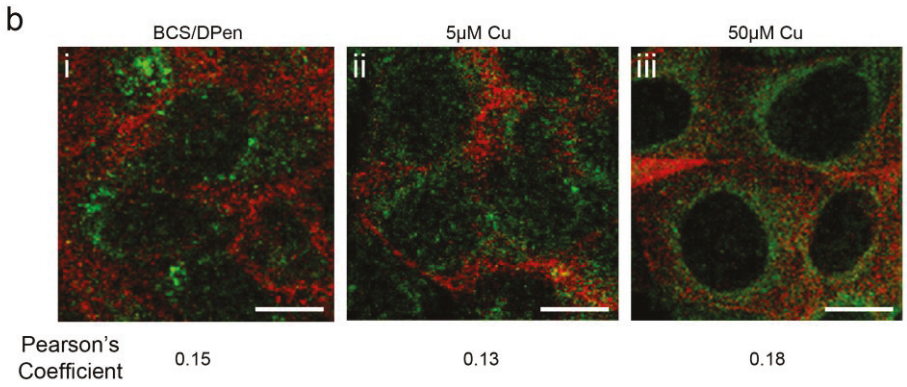
Proteins interacting with ATP7B were immunoprecipitated using ATP7B antibody as a bait and then separated by SDS-PAGE. Probing the Western blot with human Cp antibody revealed a holo-Cp band of 95 kDa, indicating that there was an interaction between Cp and ATP7B (Fig 4.6a). A copper treated whole cell lysate (lane 2), human milk (lane 3) and human serum (lane 4) were used as positive controls to identify the holo-Cp band.

To establish whether there was an interaction between Cp and ATP7A, copper treated PMC42-LA cells were analysed by dual labelled immunofluorescence. Pearson's co-efficient analysis determined that there was no co-localisation of Cp and ATP7A in cells treated with BCS/DPen (0.15), 5 μM copper (0.13) and 50 μM copper (0.18) (Fig 4.6b). Values close to zero indicate no co-localisation. Co-Ip was also performed using the ATP7A antibody, however no interaction with Cp was detected (data not shown).

Figure 4.6 Interactions between Cp and the two ATPases; ATP7A and ATP7B

(a) Western blot of co-immunoprecipitation (Co-Ip) of Cp with ATP7B. Holo-Cp was isolated from whole cell lysates by Co-Ip with ATP7B (lane 1). Whole cell lysate (lane 2), human milk (lane 3) and human serum (lane 4) were used as positive controls. (b) Co-immunolocalisation of ATP7A (green) and Cp (red) in PMC42-LA cells treated with BCS/DPen (i), 5 μM copper (ii) and 50 μM copper (iii). Pearson's coefficient values indicate degree of colocalisation of the two proteins. Scale bar = 10 μm.


a
Apo-Cp
Holo-Cp
132 kDa
95 kDa
Co-Ip
Lysate
Milk
Serum


b
BCS/DPen
5μM Cu
50μM Cu
i
ii
iii
Pearson's Coefficient
0.15
0.13
0.18

4.3 Discussion

Previous studies have focused on ceruloplasmin (Cp) found in the brain, liver and blood, but little has been reported in relation to Cp in the mammary gland and in the milk secreted during lactation. While Cp is known to be present in the mammary gland and secreted in the milk, its complete role is yet to be elucidated.

Cp mRNA has previously been detected in HC11 mammary epithelial cultured cells (Kluger et al., 2004) as well as lactating and non lactating mammary gland tissue (Cerveza et al., 2000, Maia et al., 2007, Platonova et al., 2007). This is consistent with the data presented here for the PMC42-LA models where the expression of *Cp* mRNA was detected in the non-lactating and lactating models as well as the undifferentiated cultures. Consistent with the current study, it has been widely reported that *Cp* mRNA levels do not respond to changing copper conditions in rodents and cultured cells (Broderius et al., 2010, Chen et al., 2006, Fosset et al., 2009, Gambling et al., 2004, Gitlin et al., 1992, McArdle et al., 1990, Mercer, 2001, Mostad and Prohaska, 2011, Ranganathan et al., 2011).

The data showed that soluble Cp (sCp) was synthesised and secreted in the apo-Cp form, mainly in copper deficient cells, however there was also a small amount of apo-Cp in the lysates and secretions of the copper treated cells. Apo-Cp has no bound copper, thus no ferroxidase activity (Carrico et al., 1969, Holtzman and Gaumnitz, 1970b, Holtzman and Gaumnitz, 1970a, Kojimahara et al., 1993, Kono et al., 2006a, Sato and Gitlin, 1991), and a

physiological function of apo-Cp, if any, is yet to be determined. Apo-Cp has been found in the blood in normal physiological conditions and is the main Cp component of the blood in Wilson's disease patients, as a consequence of mutations to the ATP7B gene which prevents the incorporation of copper into Cp for the creation of the ferroxidase active holo-Cp. Rats fed copper deficient diets have copper depleted plasma as there is not enough copper available for the creation of holo-Cp in the liver and so only apo-Cp is secreted (Broderius et al., 2010, Hirano et al., 2005, Holtzman and Gaumnitz, 1970a, Kojimahara et al., 1993, Matsuda et al., 1974, Terada et al., 1995). This study further supports the argument that copper does not influence the synthesis and secretion of Cp even though it is required for ferroxidase activity of the protein (Gitlin et al., 1992, Sato and Gitlin, 1991).

Ferroxidase active holo-Cp was not detected in copper depleted cells, but was present in copper treated samples. This result showing a lack of holo-Cp in copper deficient cells is consistent with other studies that have shown a marked decrease in holo-Cp levels and Cp activity in rodents fed copper deficient diets, and cultured cells treated with copper chelators (Broderius et al., 2010, Cerklewski, 1979, Evans and Abraham, 1973, Gitlin et al., 1992, Hellman et al., 2002a, Ranganathan et al., 2011, Wang and Weinman, 2004). The 50 μM copper (representative of lactational copper serum levels) treated cells had no more holo-Cp that the 5 μM copper (representative copper levels of the resting system) treated cells, indicating that there is a limit to the amount of copper that can be incorporated into Cp. This limit is reached at levels similar to basal physiological copper levels and so further metal increases will not result in a greater efflux of copper loaded Cp. Additional

intracellular copper within the cells is likely to be stored in MT, a copper chaperone, as described in chapter 3. These mechanisms may prevent excess copper being secreted into milk which would be detrimental to a suckling infant. This is consistent with previous data where serum holo-Cp levels were not elevated in rats fed high copper diets (Bauerly et al., 2005, Cerklewski, 1979, Gambling et al., 2004, Jenkins and Hidiroglou, 1989, Ranganathan et al., 2011). Cp contains 85% of the copper in milk and as lactation progresses, Cp activity and also milk copper levels decline (Albera and Kankofer, 2009, Cerveza et al., 2000, Keen et al., 1982, Platonova et al., 2007, Tsymbalenko et al., 2009). This indicates that Cp plays an important role in regulating copper efflux from the mammary epithelial cells into milk.

The addition of lactational hormones had a significant influence on the transcription and secretion of apo and holo-Cp, but not the intracellular levels of sCp protein. This is likely due to hormones inducing transcription which is balanced by a hormonally regulated increase in secretion, thus preventing a net change in total sCp within the cells. Potential estrogen and progesterone binding sites have been identified in the 5' region of the Cp gene, although functional analysis of these sites has not been performed (Tsymbalenko et al., 2009). However, estrogen, dexamethasone and insulin have all been shown previously to increase levels of *Cp* mRNA in rat livers and HepG2 liver cells, which led to increased Cp secretion and serum Cp activity (Ani and Moshtaghie, 1990, Clemente et al., 1992, Fitch et al., 1999, Middleton and Linder, 1993, Seshadri et al., 2002, Weiner and Cousins, 1983). While estrogen has been shown previously to affect Cp levels, the likely cause of the increased transcription and secretion in the PMC42-LA cells is

dexamethasone and/or insulin given that they are part of the final three day treatment of the cells whereas estrogen is administered prior to this, along with progesterone. It has been shown *in vivo* that estrogen withdrawal is one of the instigating factors, along with the withdrawal of progesterone, involved in initiating the secretion of milk during lactation, hence its position in the sequence of our treatments (Neville et al., 2002, Pang and Hartmann, 2007, Rillema, 1994). The hormonal response observed is likely to be independent of prolactin, as there was no difference between the lactational and suckled models which differ in the prolactin level.

The observed localisation of Cp is consistent with its vesicular transport to the membrane from the TGN via the secretory pathway (Schaefer et al., 1999a, Yanagimoto et al., 2011). Copper had no effect on the distribution of Cp in the cells, which is consistent with secretion of Cp being unresponsive to varying copper levels. Hormones also had no effect on the localisation of Cp. It is likely that the majority of Cp observed in these cells is sCp, based on our whole cell lysate work which did not show a distinct glycosylphosphatidylinositol-linked Cp (GPI-Cp) band. Past studies show similar distribution of sCp and GPI-Cp in cultured cells (Hida et al., 2010, Kono et al., 2006a, Kono et al., 2007, Kono et al., 2010, Mani et al., 2004, Patel et al., 2000, Persichini et al., 2010).

Cp has a variety of roles in different cells. It is likely that its role in mammary epithelial cells is related to the transport of copper into milk to nourish the suckling infant and also to oxidise iron, perhaps enabling the iron to bind to lactoferrin. Lactoferrin may have a role in providing iron to the suckling infant

for nutrition (Frazer et al., 2011, Leong and Lonnerdal, 2005), however lactoferrin has also been shown to have bactericidal activity in milk (Bullen et al., 1972, Frazer et al., 2011, Wooten et al., 1996) which occurs as a consequence of its sequestration of iron, therefore depriving bacteria of the iron they require to survive (Collard, 2009, Dorea, 2000, González-Chávez et al., 2009). If host protection is a key role for lactoferrin then it is vitally important for Cp to be secreted in its ferroxidase active form so it can convert Fe (II) to Fe (III), thus enabling lactoferrin to bind iron. This would inhibit bacterial colony formation in the gut of the developing infant, which is known to be less acidic and have decreased secretion of proteolytic enzymes which creates an ideal environment for bacterial colonisation (Davidson and Lonnerdal, 1988, Sokolov et al., 2007). Research has also confirmed an interaction between Cp and lactoferrin (Ha-Duong et al., 2010, Sabatucci et al., 2007, Sokolov et al., 2006, Zakharova et al., 2000) with one research group indicating that the majority of milk Cp is bound to lactoferrin and that this binding enhances Cp's ability to oxidise the redox active ferrous iron and scavange superoxide anion free radicals (Goldstein et al., 1979, Sokolov et al., 2009, Sokolov et al., 2005). The iron and copper levels in milk are considered lower than adequate for neonatal dietary consumption. However the levels in milk may still provide vital nutrition so that the infant does not need to rely solely on its liver stores of copper and iron in the first months of neonatal growth (Dorea, 2000). It has been shown that iron loaded lactoferrin binds to the intestinal brush border, presumably for the absorption of iron, while the copper from holo-Cp has been detected in the organs of suckling infants (Davidson and Lonnerdal, 1988, Kawakami and Lonnerdal, 1991, Linder et al., 1998, Platonova et al., 2007, Wooten et al., 1996). These data

provide evidence that Cp and lactoferrin are dietary sources of trace elements.

The research presented in this chapter has for the first time reported the presence of GPI-Cp mRNA and protein in mammary epithelial cells. GPI-Cp is a product of *Cp* mRNA splicing, which enables *GPI-Cp* to be distinguished from the secreted *Cp* mRNA sequence (Banha et al., 2008, Patel et al., 2000). GPI-Cp has been previously observed in a number of cell types and tissues including lymphocyte, kidney, spleen, liver and also in sertoli, leptomeningeal and astrocyte brain cells (Banha et al., 2008, Fortna et al., 1999, Kono et al., 2010, Mittal et al., 2003, Mostad and Prohaska, 2011, Patel and David, 1997), though has not been reported in the mammary gland.

GPI-Cp is a 135 kDa membrane bound form of Cp (Kono et al., 2006b, Mostad and Prohaska, 2011, Patel et al., 2000) protein which was detected by surface biotinylation indicating its presence at the plasma membrane. GPI-Cp could not be detected in whole cell lysates suggesting that its levels are significantly lower than sCp in mammary epithelial cells. Copper treatment had no major influence on the level of GPI-Cp protein found at the plasma membrane. A previous study reported a reduction in GPI-Cp protein levels in C6 glioma cells in response to BCS (de Domenico et al., 2007). The data described here was consistent with the glioma cells, as the GPI-Cp levels reduced slightly upon copper depletion, however the differences were not statistically significant. A possible reason for the discrepancy is likely due to differences in cell type between the brain and mammary gland.

Currently no work has been published on the effects of lactation or lactational hormones on the levels of GPI-Cp. Previously it was shown that the holo and apo forms of GPI-Cp can be distinguished from each other in glioma cells and that the levels of each vary in response to copper (di Patti et al., 2009, Kono et al., 2006a, Kono et al., 2010) similar to the sCp shown in this chapter. Unfortunately the final elution of the biotinylated protein required the use of DTT and while no heat was applied, a step required to denature human Cp (Kim et al., 2009, Sato and Gitlin, 1991), it appeared that the GPI-Cp has been completely denatured. Some forms of Cp denature more readily than others, as was demonstrated with rat Cp compared to human Cp (Sato and Gitlin, 1991, Terada et al., 1995) and perhaps GPI-Cp denatures more easily than sCp. Had GPI-Cp not been denatured, it is possible that a similar expression pattern to that of the sCp protein may have been observed in response to copper. Lactational hormones had no effect upon the expression of GPI-Cp in the mammary epithelial cells.

Current roles attributed to GPI-Cp include the oxidation of iron on account of its oxidase activity, and a role in iron efflux from cells (Jeong and David, 2003, Kono et al., 2006a). GPI-Cp was shown to be important in aiding the efflux of iron through ferroportin (de Domenico et al., 2007), a protein also found to be present in the mammary gland (Leong and Lonnerdal, 2005). Perhaps GPI-Cp's main role is to aid the efflux of iron into the milk secretions via ferroportin, which transports Fe (II). GPI-Cp may then oxidise the iron to Fe (III) in a similar fashion to sCp, so that the iron is then able to bind to lactoferrin. However as this is the first time that GPI-Cp expression has been identified in mammary epithelial cells, it is yet to be determined whether there

is a direct interaction between GPI-Cp and lactoferrin. This role could be particularly useful if the majority of sCp formed a permanent complex with lactoferrin (Sokolov et al., 2009) as GPI-Cp would enable more of the available lactoferrin in milk, which has been shown to be 75-88% apo-lactoferrin (Sokolov et al., 2005), to bind additional iron. GPI-Cp may also work in conjunction with other enzymes like hephaestin or zyklopen. Hephaestin and zyklopen are two other membrane bound multi-copper ferroxidases, however only zyklopen has been detected in mammary glands (Chen et al 2010).

Analysis of PMC42-LA cells confirmed an interaction between ATP7B and Cp in the mammary gland consistent with ATP7B's role in delivering copper to Cp for secretion into milk. No interaction could be identified between ATP7A and Cp by co-localisation or co-immunoprecipitation analysis. While this suggests that ATP7A is not involved in delivery of copper into milk via Cp, this also may be a consequence of ATP7B being fully functional in these cells. In previous studies implicating ATP7A in the delivery of copper to Cp, ATP7B has not been present (Barnes et al., 2005, White et al., 2009a). A fully functioning ATP7B protein may dominate the process leaving any Cp-ATP7A interaction undetectable by the methods used. If ATP7A does not deliver copper to Cp, it does leave open the question as to how copper is secreted from the mammary gland in *tx* mice. However it has been suggested that the remaining copper not bound to Cp, may be bound to other copper dependent enzymes like superoxide dismutase (Tsymbalenko et al., 2009).

4.4 Conclusion

This research reports for the first time that GPI-Cp is present on the plasma membrane of mammary epithelial cells. It also demonstrates that sCp is secreted from these cells in both the apo and holo forms. The level of ferroxidase active holo-Cp is dependent on the availability of copper and the total level of secretion is influenced by the presence of lactational hormones. This research also identified a direct interaction between ATP7B and Cp in the mammary gland, consistent with ATP7B's previously ascribed function in delivering copper to Cp for secretion into milk. These data provide new insight into the role of Cp in mammary epithelial cells, particularly during lactation. The knowledge that copper modulates the levels of bioactive holo-Cp secreted into milk levels could be used to advance research into neonatal nutritional supplementation, while the involvement of both sCp and GPI-Cp in enabling lactoferrin to sequester iron is also important for improving neonatal health.

CHAPTER 5

ATP7A overexpression disrupts copper homeostasis in cultured PMC42-LA mammary epithelial cells

5.1 Introduction

Cellular copper and iron homeostasis is regulated by influx and efflux proteins including CTR1, ATP7A, ATP7B, ceruloplasmin (Cp), transferrin receptor 1 (TfR) and β2-microglobulin (β2M). To summarise the role of the different proteins, CTR1 plays an important role in copper uptake into the cells. ATOX1 then delivers it to the ATPases, ATP7A and ATP7B. The ATPases distribute copper to other copper dependent enzymes including Cp, or efflux excess copper from the cells (Bhatt et al., 2010, Enns, 2001, Lutsenko et al., 2007a, Mayle et al., 2012, Prohaska, 2008). Holo-Cp, the copper loaded form, has ferroxidase activity and plays an important role in the oxidation of Fe (II) to Fe (III) as well as the influx and efflux of iron, depending on cell type (Attieh et al., 1999, Chang et al., 2007, de Domenico et al., 2007, Harris et al., 1999, Jeong and David, 2003, Qian et al., 2001, Sarkar et al., 2003, Terada et al., 1995, Tsymbalenko et al., 2009, Wooten et al., 1996). TfR also plays an important role in iron uptake into cells, binding and endocytosing iron loaded transferrin with the assistance of β2M (Bhatt et al., 2009, Bhatt et al., 2010, Enns, 2001, Vecchi et al., 2010). When copper levels are elevated within cells, metallothionein 1 and 2 (MT) are used as long term storage molecules, to protect the cells from toxicity (Coyle et al., 2002, Mehta et al., 2006, Prohaska and Gybina, 2004, Sutherland and Stillman, 2011).

Copper transporters have been extensively studied, identifying their individual responses to copper. However there are few reports of the analysis of copper transporters in the context of a whole system. Copper efflux in

most tissues is carried out by ATP7A (Vulpe et al., 1993), while the liver and brain express ATP7B in conjunction with Cp to transport copper from the cells (Bull et al., 1993, Terada et al., 1998). The mammary gland is unusual in that it expresses both ATPases and Cp, thus giving these tissues the unusual characteristic of having three copper efflux proteins (Ackland et al., 1999, Linder et al., 1998, Llanos et al., 2008, Michalczyk et al., 2008, Tsymbalenko et al., 2009, Yang et al., 1990). Previously, ATP7A and ATP7B have partially been analysed in the PMC42-LA mammary epithelial cell line (Ackland et al., 1997, Michalczyk et al., 2008).

In this chapter the mammary epithelial cell line PMC42-LA was used to investigate the consequences of overexpression of ATP7A and provide novel insight into the copper homeostasis network of proteins. To investigate the interactions between the different components of copper homeostasis and how these copper proteins are regulated by intracellular copper, the copper efflux protein ATP7A was overexpressed in the PMC42-LA cells with the intention of reducing intracellular copper levels. The hypothesis is that this disruption in copper homeostasis will impact upon the other copper transporting proteins and that they may be regulated to maintain intracellular copper and maintain an adequate supply of copper to the milk. In this chapter the mammary epithelial cell line PMC42-LA was used to investigate the consequences of overexpression of ATP7A and provide novel insight into the copper homeostasis network of proteins.

It is proposed that the responses of CTR1 and MT occur to maintain copper balance in the cells, however the changes to β2M and Cp indicate that

disruption of copper homeostasis can have a more widespread impact than initially anticipated. While these two proteins are known to be influenced by copper, they have roles associated with iron uptake and metabolism. This research has demonstrated the capacity of cells to adjust to disruptions in homeostasis particularly in a system containing three copper efflux proteins; ATP7A, ATP7B and Cp. It shows that the finely tuned homeostatic mechanisms operate to balance disturbances to copper fluxes.

5.2 Results

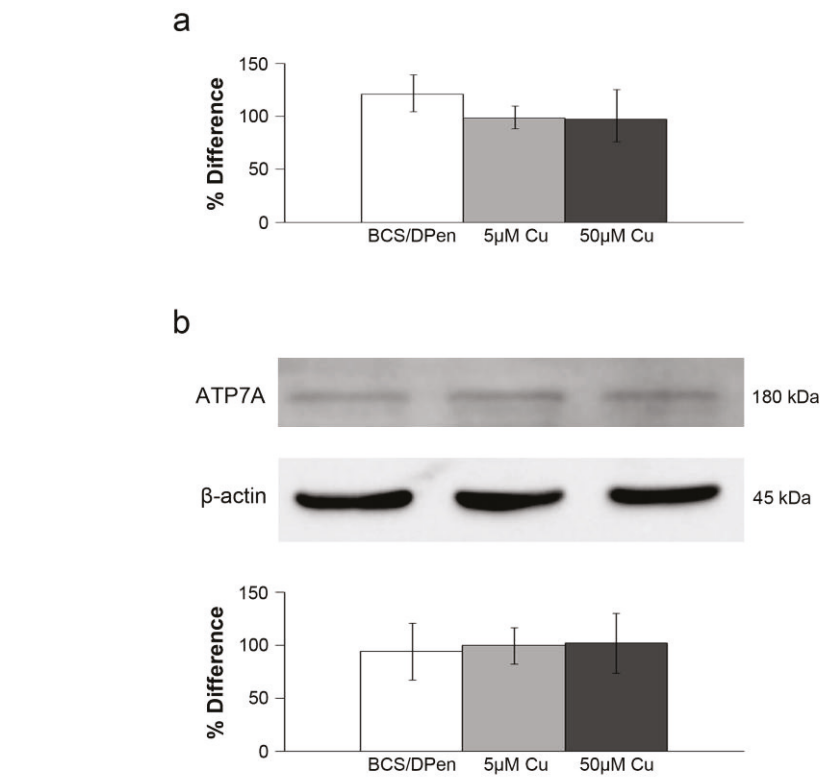
5.2.1 Copper did not affect mRNA or protein levels, but influenced the localisation of endogenous ATP7A protein in PMC42-LA cells

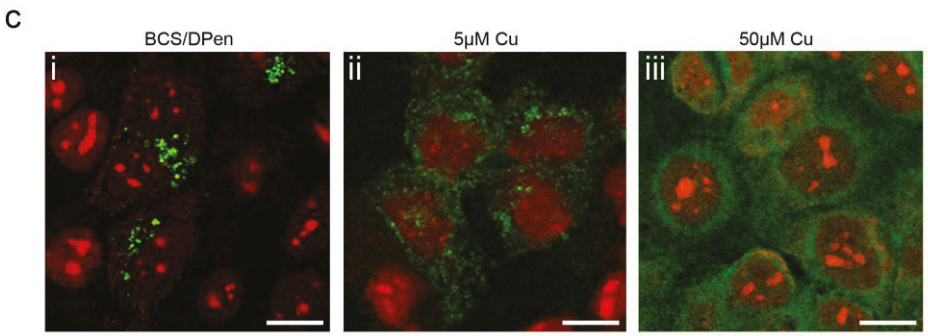
Undifferentiated PMC42-LA cells were grown in culture flasks and treated with varying concentrations of copper to determine the effect on endogenous ATP7A expression. qRT-PCR analysis showed that there was no significant difference ($p>0.05$) in mRNA levels between cells grown in the presence of copper or copper chelators (Fig 5.1a). This trend was mirrored in the analysis of ATP7A protein levels, determined via Western blotting (Fig 5.1b). A single band at approximately 180 kDa was detected in cells treated with copper and copper chelators, however densitometric analysis confirmed there were no significant changes in its intensity ($p>0.05$).

To determine the effect of copper on ATP7A localisation, PMC42-LA cells were grown on glass coverslips and analysed by immunofluorescence confocal microscopy (Fig 5.1c). Reducing copper levels with BCS/DPen (i) resulted in accumulation of ATP7A in the perinuclear region. With increasing copper, ATP7A became more dispersed throughout the cytoplasm. The 5 μM copper treatment (ii) caused ATP7A to move towards the plasma membrane, with some protein remained in the perinuclear region of the cells. Treatment with 50 μM copper (iii) resulted in complete dispersion of ATP7A protein throughout the cytoplasm, with no distinct aggregation remaining in the perinuclear region.

Figure 5.1 ATP7A levels and localisation in response to copper in undifferentiated PMC42-LA cells

(a) Relative *ATP7A* mRNA levels of 50 μM Cu and BCS/Dpen treatments compared to that of the 5 μM Cu treatment. The bar graph shows mRNA mean percentages ±SD from three independent experiments normalised against *β-actin* obtained by qRT-PCR. (b) Representative Western blot analysis of 60μg of undifferentiated PMC42-LA cell extracts. Lane 1, BCS/DPen treatment; lane 2, 5 μM copper treatment; lane 3, 50 μM copper treatment. β-actin was used as a loading control. Densitometric analysis shows the intensity of the ATP7A Western blot bands expressed as a mean percentage of arbitrary units ±SD compared to the 5 μM copper treated sample normalised against β-actin. This figure shows representative results of three independent experiments. (c) Immunolocalisation of ATP7A (green) in undifferentiated PMC42-LA cells grown on glass coverslips treated with BCS/Dpen (i), 5 μM copper (ii) and 50 μM copper (iii). Nuclei were stained with ethidium bromide (red). Scale bar = 10 μm.


a
150
100
50
0
% Difference
BCS/DPen
5μM Cu
50μM Cu
b
ATP7A
180 kDa
β-actin
45 kDa
150
100
50
0
% Difference
BCS/DPen
5μM Cu
50μM Cu


c
BCS/DPen
5μM Cu
50μM Cu
i
ii
iii

5.2.2 Overexpression of ATP7A in PMC42-LA cells

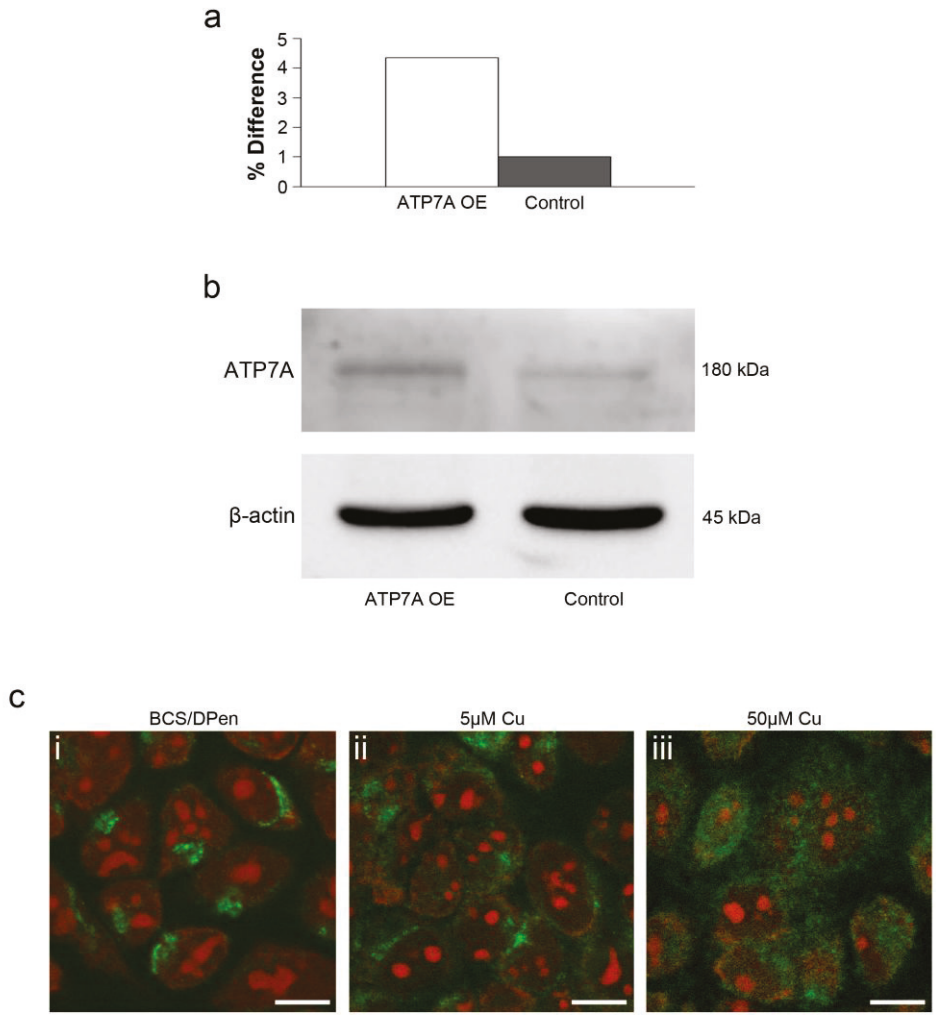
To investigate the interactions between the major copper transporters, a previously developed *ATP7A* overexpression plasmid, pCMB344 (Ke et al., 2006) was transfected into PMC42-LA cells. *ATP7A*-transfected cells had a 435% increase in relative levels of *ATP7A* mRNA compared with cells transfected with an empty vector (Fig 5.2a). The empty vector cells will be referred to as "control cells" for the remainder of the thesis. Western blot analysis showed increased levels of ATP7A protein (Fig 5.2b). The addition and depletion of copper had no influence on the levels of ATP7A mRNA and protein in the ATP7A overexpressing cells (data not shown).To confirm the exogenous ATP7A trafficked like the endogenous protein, immunolocalisation of ATP7A in the overexpressing cell line was carried out. No discernible difference was observed between the overexpressing and control cells (Fig 5.1c) under corresponding copper conditions. ATP7A in the overexpressing cells was located in the perinuclear region in the presence of copper chelators BCS and DPen, while the addition of copper caused the protein to disperse throughout the cell towards the membrane (Fig 5.2c) which is characteristic of the endogenous protein.

5.2.3 ATP7A overexpression and copper treatment did not affect the localisation of copper transporting proteins in PMC42-LA cells

PMC42-LA cells with and without the ATP7A overexpressing construct were grown in culture flasks and treated with copper and copper chelators to investigate how copper transporters respond to altered copper homeostasis. Responses to copper and copper chelators were measured in ATP7A overexpressing cells. Treatment with 5 μM copper was used to simulate

Figure 5.2 Confirmation of ATP7A overexpression and functionality in PMC42-LA cells

(a) Relative *ATP7A* mRNA levels isolated from ATP7A overexpressing cells (lane 1) compared to control cells (lane 2). The bar graph shows mean percentage of relative mRNA normalised against *β-actin* obtained by qRT-PCR. (b) Representative ATP7A Western blot analysis of 60 µg ATP7A overexpressing cell extract (lane 1) compared to control (lane 2). β-actin was used as a loading control. (c) Immunolocalisation of ATP7A (green) in ATP7A overexpressing PMC42-LA cells treated with BCS/DPen (i), 5 µM copper (ii) and 50 µM copper (iii). Nuclei were stained with ethidium bromide (red). Scale bar = 10 µm.


a
% Difference
5
4
3
2
1
0
ATP7A OE
Control
b
ATP7A
180 kDa
β-actin
45 kDa
ATP7A OE
Control
c
BCS/DPen
5μM Cu
50μM Cu
i
ii
iii

normal copper levels in the exchangeable pool of copper in blood plasma which is in the range of 2.7 and 7.7 μM (Linder, 1991, Linder, 2002). The 50 μM copper treatment demonstrated the effect of high copper levels. Copper chelators were added to determine the effect of copper deficiency in the mammary epithelial cells. ATP7B was shown to disperse from the perinuclear region towards the membrane with the addition of copper, while CTR1 and Cp displayed a granular distribution throughout the cells under all conditions. The overexpression of ATP7A in these cells had no influence on the localisation of ATP7B, CTR1 or Cp in copper depleted or repleted conditions (Fig 5.3).

5.2.4 ATP7A overexpression increased the transcription of *Cp*, but did not affect *β2M*, *CTR1*, *ATOX1*, or *ATP7B* mRNA levels

To measure the transcriptional responses to altered copper homeostasis, the mRNA levels of several copper related genes were analysed by qRT-PCR in response to ATP7A overexpression and different copper conditions. There was a significant increase in the total *Cp* mRNA levels in ATP7A overexpressing cells (BCS/Dpen – 571.2 ± 81.6%, 5 μM copper – 646.5 ± 71.6%, 50 μM copper – 765.3 ± 113.5%) compared to the control PMC42-LA cells (BCS/DPen – 102.5 ± 8.7%, 5 μM copper – 100.0 ± 16.1% and 50 μM copper 81.6 ± 6.4%) for each of the copper treatments ($p<0.05$) (Fig 5.4a). However there was no significant variation in response to copper levels. *β2M* (Fig 5.4b), *CTR1* (Fig 5.5a), *ATOX1* (Fig 5.5b) and *ATP7B* (Fig 5.5c) were not altered in ATP7A overexpressing cells compared to controls or in response to copper levels ($p>0.05$).

Figure 5.3 ATP7B, CTR1 and Cp localisation in undifferentiated PMC42-LA cells

Immunolocalisation of ATP7B, CTR1 and Cp (green) in ATP7A overexpressing PMC42-LA cells (ATP7B (a), CTR1 (c) and Cp (d)) and also PMC42-LA control cells (ATP7B (b)) treated with BCS/DPen (i), 5 μM copper (ii) and 50 μM copper (iii). Nuclei were stained with ethidium bromide (red). Scale bar = 10 μm.

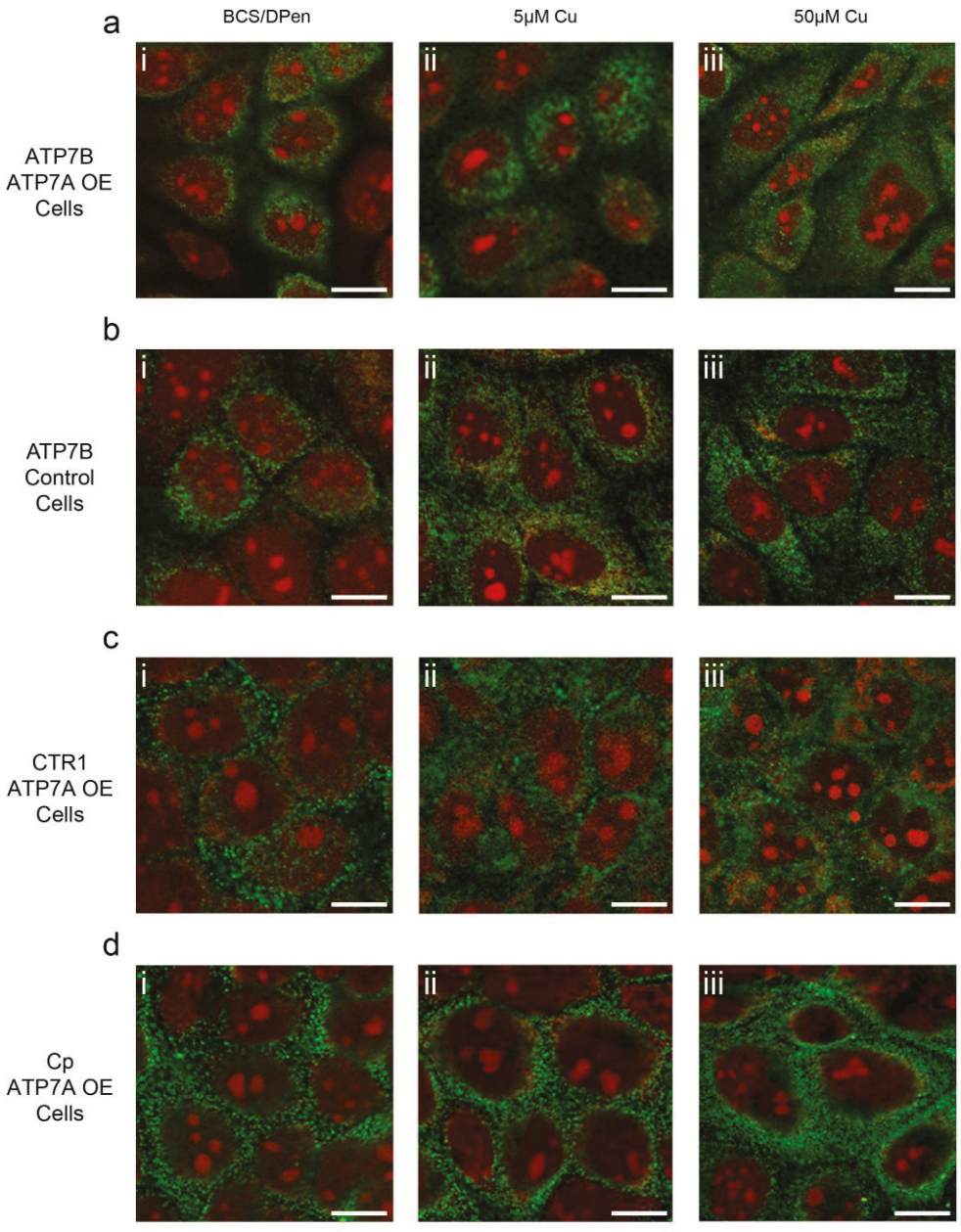

a
BCS/DPen
5μM Cu
50μM Cu
ATP7B
ATP7A OE
Cells
i
ii
iii
b
ATP7B
Control
Cells
c
CTR1
ATP7A OE
Cells
d
Cp
ATP7A OE
Cells

Figure 5.4 *Cp* and *β2M* mRNA levels in response to ATP7A overexpression in undifferentiated PMC42-LA cells

The relative levels of *Cp* (a) and *β2M* (b) mRNA isolated from ATP7A overexpressing cells (lanes 1-3) compared to control (lanes 4-6) and treated with BCS/DPen (lanes 1 and 4), 5 μM copper (lanes 2 and 5) and 50 μM copper (lanes 3 and 6) were measured. The bar graphs show mean percentages of relative mRNA ±SD normalised against *β-actin* obtained by qRT-PCR. Statistical variation is indicated by different letters above graph bars ($p<0.05$).

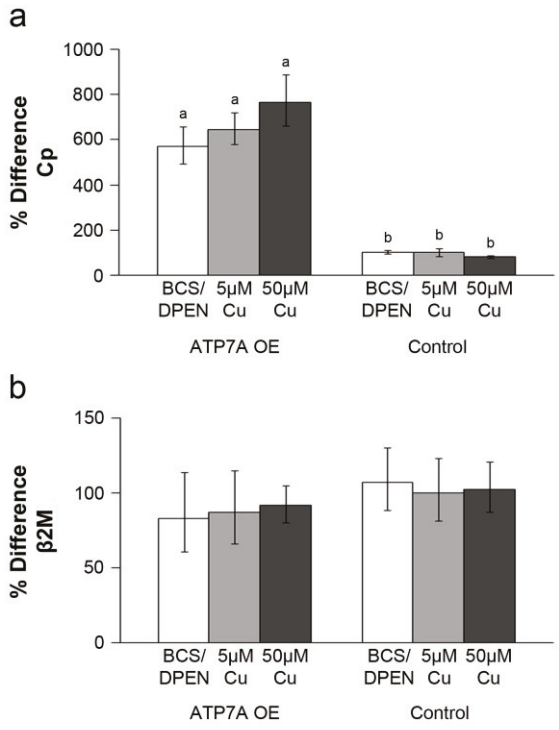
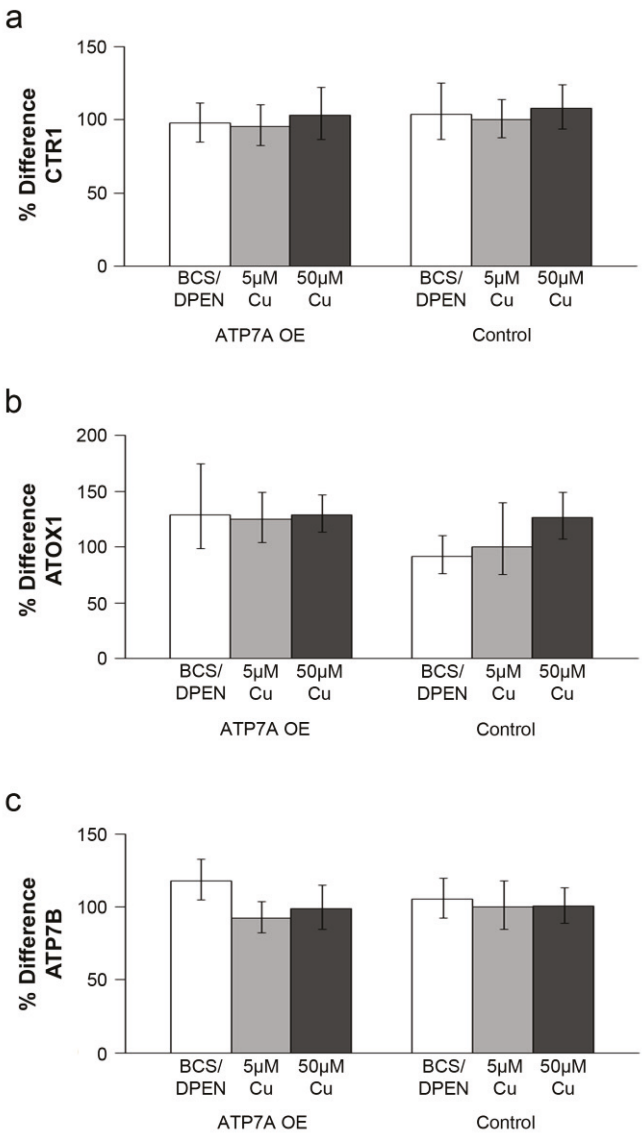

a
% Difference Cp
1000
800
600
400
200
0
a
a
a
b
b
b
BCS/ DPEN
5μM Cu
50μM Cu
BCS/ DPEN
5μM Cu
50μM Cu
ATP7A OE
Control
b
% Difference β2M
150
100
50
0
BCS/ DPEN
5μM Cu
50μM Cu
BCS/ DPEN
5μM Cu
50μM Cu
ATP7A OE
Control

Figure 5.5 The levels of *CTR1*, *ATOX1* and *ATP7B* mRNA in ATP7A overexpression cells

Relative levels of *CTR1* (a), *ATOX1* (b) and *ATP7B* (c) mRNA isolated from ATP7A overexpressing cells (lanes 1-3) compared to control (lanes 4-6), and treated with BCS/DPen (lanes 1 and 4), 5 μM copper (lanes 2 and 5) and 50 μM copper (lanes 3 and 6). The bar graphs show mean percentage of relative mRNA ±SD normalised against *β-actin* obtained by qRT-PCR.


a
% Difference CTR1
150
100
50
0
BCS/ DPEN
5μM Cu
50μM Cu
BCS/ DPEN
5μM Cu
50μM Cu
ATP7A OE
Control
b
% Difference ATOX1
200
150
100
50
0
BCS/ DPEN
5μM Cu
50μM Cu
BCS/ DPEN
5μM Cu
50μM Cu
ATP7A OE
Control
c
% Difference ATP7B
150
100
50
0
BCS/ DPEN
5μM Cu
50μM Cu
BCS/ DPEN
5μM Cu
50μM Cu
ATP7A OE
Control

5.2.5 Increased expression of ATP7A influenced the protein levels of CTR1, Cp, MT and β2M, while ATOX1 and ATP7B levels remained unaltered

Total protein levels of CTR1, Cp, MT, β2M, ATOX1 and ATP7B were measured by Western blot analysis. The 35 kDa CTR1 monomer protein levels were reduced by exposure to elevated copper conditions in ATP7A overexpressing cells. It was also observed that ATP7A overexpressing cells expressed significantly more CTR1 protein in each copper condition (BCS/DPen 377.0% ± 39.7%, 5 μM copper 161.5% ± 7.5% and 50 μM copper 119.7% ± 6.9%) compared to the corresponding control sample (BCS/DPen 239.6 ± 25.6%, 5 μM copper 100.0 ± 15.1% and 50 μM copper 67.1 ± 12.2%) ($p<0.05$) (Fig 5.6). Additionally, the amount of CTR1 protein present at the plasma membrane as determined by surface biotinylation, correlates with the total CTR1 protein levels. The protein levels at the membrane decreased in response to high copper treatment, but overall were still higher in response to ATP7A overexpression (Fig 5.9).

ATOX1 and ATP7B protein expression were analysed in response to copper treatments and ATP7A overexpression. These factors did not have any impact on the levels of these two proteins ($p>0.05$) (Fig 5.7a).

Bands of 132 kDa, 130 kDa and 95 kDa corresponing to apo-Cp, unglycosylated Cp (Ug-Cp) and holo-Cp were detected and their intensity was significantly altered by ATP7A overexpression (Fig 5.6). Holo-Cp was present predominantly in the copper treated samples, and its expression was significantly higher in the ATP7A overexpressing cells (BCS/DPen 118.6 ±

19.8%, 5 μM copper 261.6 ± 42.5% and 50 μM copper 324.1 ± 51.4%) compared to the controls (BCS/DPen 68.1 ± 21.1%, 5 μM copper 100.0 ± 20.6% and 50 μM copper 113.0 ± 14.8%) ($p<0.05$) (Fig 5.6). Apo-Cp, present mainly in copper chelated cells, was significantly elevated in the ATP7A overexpressing cultures (BCS/DPen 253.5 ± 51.7%, 5 μM copper 157.2 ± 29.8% and 50 μM copper 162.1 ± 37.0%) compared to each respective control sample (BCS/DPen 153.5± 42.3%, 5 μM copper 100.0 ± 19.0% and 50 μM copper 89.1 ± 19.4%) ($p<0.05$) (Fig 5.6). The differences observed in the lysates were strongly reflected in the Cp protein that was secreted from these cells. Holo-Cp secretion was shown to increase with copper, while apo-Cp decreased and while the trends were the same between the two cells types, the ATP7A overexpressing cells secreted significantly more apo-Cp and holo-Cp in each of the copper conditions (Fig 5.8). Glycosylphosphatidylinositol-linked Cp (GPI-Cp) detected by cell surface biotinylation also showed a statistically significant increase in response to ATP7A overexpression ($p<0.05$) (Fig 5.9), however was unaltered in response to copper levels.

MT was undetected in BCS/DPen and 5 μM copper treated ATP7A overexpressing and control PMC42-LA cells, however upon treatment with 50 μM copper, a MT band became clearly visible. The levels observed in the ATP7A overexpressing cells were significantly lower than the control cells being only 49.7 ± 10.6% that of the control PMC42-LA cells (100.0 ± 13.0%) ($p<0.05$) (Fig 5.6).

β2M showed a significant increase in protein levels in response to ATP7A overexpression in each of the BCS/DPen (ATP7A-OE 217.0 ± 45.5%, control 79.2 ± 17.1%), 5 μM (ATP7A-OE 219.9 ± 74.7%, control 100.0 ± 31.4%) and 50 μM copper treatments (ATP7A-OE 240.4 ± 89.8%, control 106.9 ± 30.2%) ($p<0.05$) (Fig 5.6). However the addition of copper or copper chelators had no influence on the levels of β2M in either cell line ($p>0.05$).

The role of TfR in iron uptake is influenced by β2M, however unlike β2M, the TfR protein showed no significant difference in response to ATP7A overexpression. The copper treatments, both supplementation and depletion also had no influence on the protein levels of TfR in PMC42-LA cells (Fig 5.7a).

Surface biotinylation was performed on PMC42-LA cells to determine the levels of ATP7A present at the plasma membrane. ATP7A levels increased in response to copper in ATP7A overexpressing (BCS/DPen 58.9 ± 35.1%, 5 μM copper 126.5 ± 70.4% and 50 μM copper 147.9 ± 33.1%) and control PMC42-LA cells (BCS/DPen 26.4 ± 16.2%, 5 μM copper 100.0 ± 7.9% and 50 μM copper 89.4 ± 6.3%). Statistically significant differences were observed between the BCS/DPen treated samples and the 50 μM copper treated samples in both cell lines ($p<0.05$), however no difference was observed between the 5 μM and 50 μM copper treated samples ($p>0.05$). The level of ATP7A at the membrane in the ATP7A overexpressing cells was higher than in the control cells for each of the copper treatments indicating that the transgene is functional and moving to the membrane, however these

differences were only statistically significant between the 50 μM copper treated cells ($p<0.05$) (Fig 5.9).

Surface biotinylation was also performed on ATP7B, however no protein was detected at the membrane in response to any of the variable copper conditions in either the ATP7A overexpressing cells or control PMC42-LA cells (data not shown).

5.2.6 Cp protein expression was not influenced by iron treatment in PMC42-LA cells

Cp has a role in oxidising iron, enabling it to be transported in and out of cells. It has been shown that iron levels can influence the levels of Cp. Cultured PMC42-LA cells were treated with iron and Cp protein levels were measured. Cells treated with 100, 300, 500 and 1000 μM iron showed no significant variation in Cp protein levels compared to that of the non-treated control ($p>0.05$) (Fig 5.7b).

5.2.7 ATP7A overexpression decreased intracellular copper levels of copper loaded cells, while iron levels were unaffected

Atomic absorption spectroscopy (AAS) was performed to determine intracellular metal levels in response to copper treatment and ATP7A overexpression. ATP7A overexpressing cells had similar levels of copper (BCS/DPen – 26.0 ± 1.8 ng/mg protein, 5 μM copper – 34.7 ± 8.8 ng/mg protein) as the control cells (BCS/DPen – 32.9 ± 7.0 ng/mg protein, 5 μM copper 27.4 ± 6.7 ng/mg protein) when exposed to basal copper levels or copper chelators ($p>0.05$). There was also little variation between these two

Figure 5.6 CTR1, MT, Cp and β2M protein levels in response to ATP7A overexpression in undifferentiated PMC42-LA cells

Representative Western blots of 60 µg of undifferentiated PMC42-LA total protein extracts showing bands for CTR1, MT, apo-Cp, holo-Cp and β2M protein. ATP7A overexpressing cells (lanes 1-3) treated with BCS/DPen, 5 µM copper and 50 µM copper are compared to control cells (lanes 4-6). β-actin was used as a loading control. Densitometric analysis of band intensity are expressed as a mean percentage of arbitrary units ±SD compared to the 5 µM copper treated control sample and normalised against β-actin. This figure shows representative results of three independent experiments. Statistical variation is indicated by different letters above graph bars ($p<0.05$).

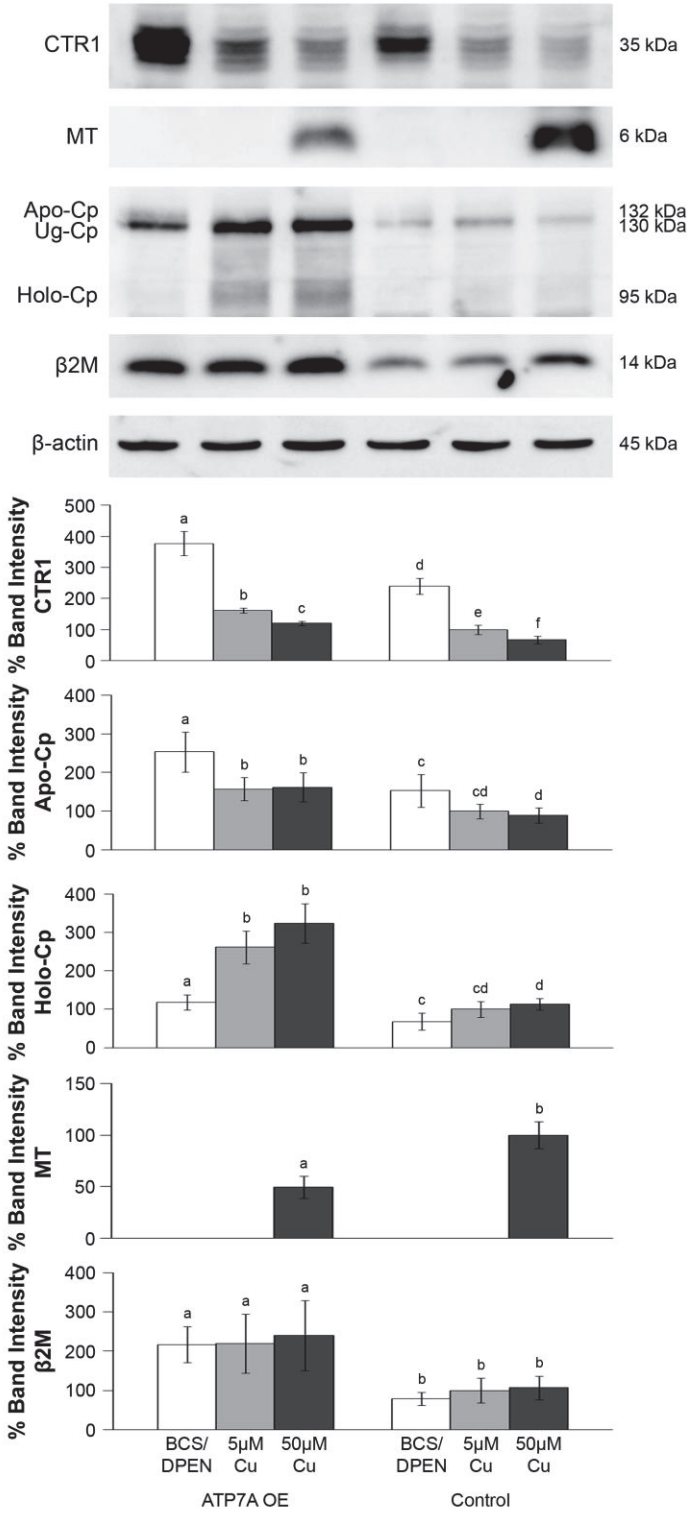

CTR1
35 kDa
MT
6 kDa
Apo-Cp
Ug-Cp
132 kDa
130 kDa
Holo-Cp
95 kDa
β2M
14 kDa
β-actin
45 kDa
% Band Intensity CTR1
% Band Intensity Apo-Cp
% Band Intensity Holo-Cp
% Band Intensity MT
% Band Intensity β2M
BCS/ DPEN
5μM Cu
50μM Cu
ATP7A OE
Control

Figure 5.7 ATP7B, ATOX1 and TfR protein levels in response to ATP7A overexpression and the influence of iron upon sCp protein expression in undifferentiated PMC42-LA cells

(a) Representative Western blots of 60 µg of undifferentiated PMC42-LA total protein extracts showing bands for ATP7B, ATOX1 and TfR protein. ATP7A overexpressing cells (lanes 1-3) treated with BCS/DPen, 5 µM copper and 50 µM copper are compared to control cells (lanes 4-6). β-actin was used as a loading control. Densitometric analysis of band intensity are expressed as a mean percentage of arbitrary units ±SD compared to the 5 µM copper treated control sample and normalised against β-actin. This figure shows representative results of three independent experiments. (b) Representative Cp Western blot analysis of 60 µg of undifferentiated PMC42-LA cell extracts treated with 0 µM, 100 µM, 300 µM, 500 µM and 1000 µM iron. Ponceau stain was used as a loading control. Densitometric analysis of band intensity are expressed as a mean percentage of arbitrary units ±SD compared to the 0 µM iron treated control sample and normalised against Ponceau. This figure shows representative results of three independent experiments.

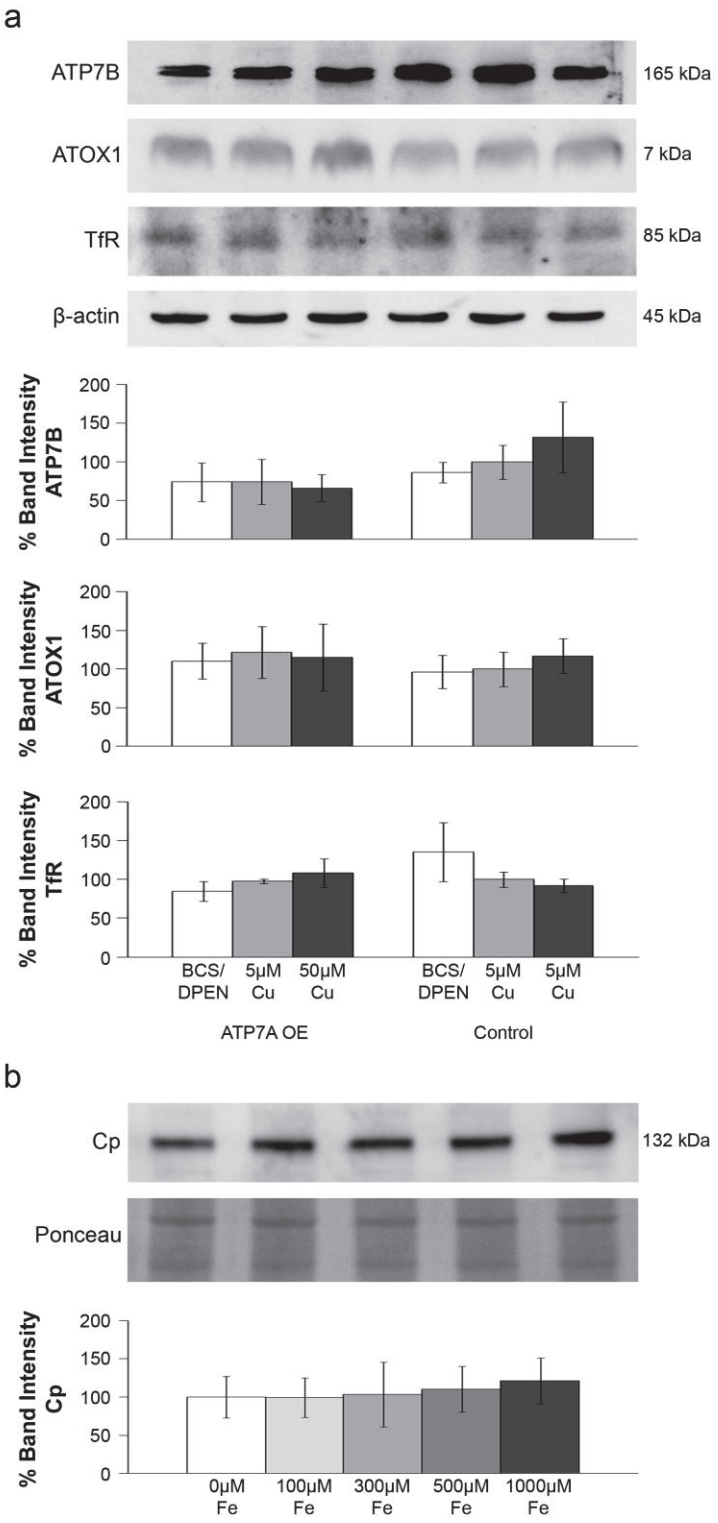

a
ATP7B
165 kDa
ATOX1
7 kDa
TfR
85 kDa
β-actin
45 kDa
% Band Intensity ATP7B
% Band Intensity ATOX1
% Band Intensity TfR
200
150
100
50
0
BCS/ DPEN
5μM Cu
50μM Cu
BCS/ DPEN
5μM Cu
5μM Cu
ATP7A OE
Control
b
Cp
132 kDa
Ponceau
% Band Intensity Cp
0μM Fe
100μM Fe
300μM Fe
500μM Fe
1000μM Fe

Figure 5.8 Secreted Cp protein levels in response to ATP7A overexpression and copper treatments

Representative Cp Western blot of 60 µg of protein obtained from the growth medium collected at the conclusion of the experiment from cultured PMC42-LA cells treated with copper and copper chelators. Lane 1, BCS/DPen treatment; lane 2, 5 µM copper treatment; lane 3, 50 µM copper treatment. Ponceau stain was used as a loading control. Densitometric analysis shows the intensity of the 132 kDa apo-Cp and 95 kDa holo-Cp bands expressed as a mean percentage of arbitrary units ±SD compared to the 5 µM copper treated control sample normalised against Ponceau S stain. This figure shows representative results of three independent experiments. Statistical variation is indicated by different letters above graph bars ($p<0.05$).

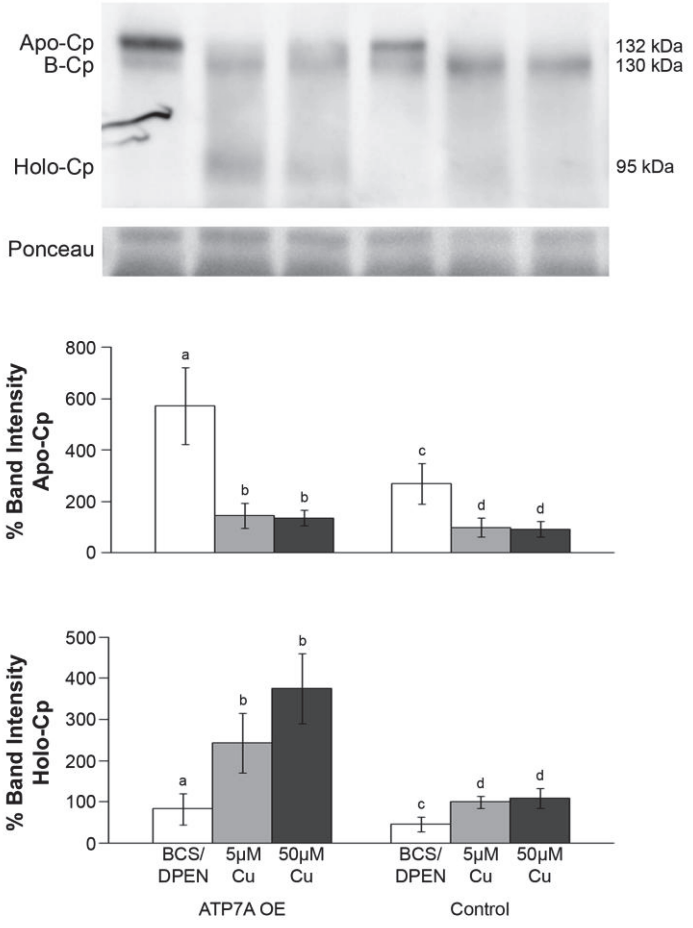
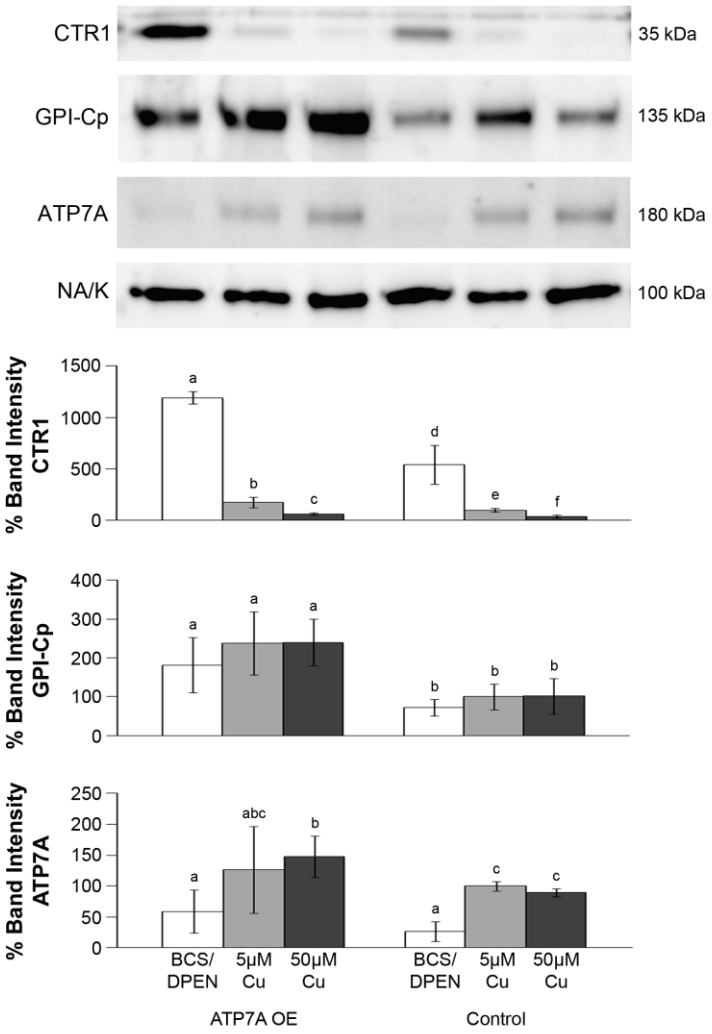

Apo-Cp
B-Cp
Holo-Cp
132 kDa
130 kDa
95 kDa
Ponceau
% Band Intensity Apo-Cp
800
600
400
200
0
a
b
b
c
d
d
% Band Intensity Holo-Cp
500
400
300
200
100
0
a
b
b
c
d
d
BCS/ DPEN
5μM Cu
50μM Cu
BCS/ DPEN
5μM Cu
50μM Cu
ATP7A OE
Control

Figure 5.9 Plasma membrane bound CTR1, GPI-Cp and ATP7A protein levels in undifferentiated PMC42-LA cells

Representative Western blot of 60 µg of extracellularly biotinylated protein extract showing bands for CTR1, GPI-Cp, and ATP7A protein, isolated from ATP7A overexpressing (lanes 1-3) and control cells (lanes 4-6) treated with BCS/DPen, 5 µM copper and 50 µM copper. Na/K ATPase was used as a loading control. Densitometric analysis of band intensity are expressed as a mean percentage of arbitrary units ±SD compared to the 5 µM copper treated control sample and normalised against Na/K ATPase. This figure shows representative results of three independent experiments. Statistical variation is indicated by different letters above graph bars ($p<0.05$).


CTR1
35 kDa
GPI-Cp
135 kDa
ATP7A
180 kDa
NA/K
100 kDa
% Band Intensity CTR1
1500
1000
500
0
a
b
c
d
e
f
% Band Intensity GPI-Cp
400
300
200
100
0
a
a
a
b
b
b
% Band Intensity ATP7A
250
200
150
100
50
0
a
abc
b
a
c
c
BCS/ DPEN
5μM Cu
50μM Cu
BCS/ DPEN
5μM Cu
50μM Cu
ATP7A OE
Control

copper conditions. Upon elevation of copper treatment to 50 μM, the cells accumulated significantly higher levels of copper ($p<0.05$). ATP7A overexpressing cells (444.6 ± 68.3 ng/mg protein) accumulated significantly less copper than the control cells (586.9 ± 33.3 ng/mg protein) consistent with the role of ATP7A as a copper efflux protein ($p<0.05$) (Fig 5.10a).

Copper and iron homeostasis have previously been shown to be linked, with the levels of one metal being affected inversely in response to the other. AAS was performed on PMC42-LA cells exposed to the various copper conditions to determine whether the observed changes in cellular copper levels affected intracellular iron levels. Iron levels for each copper treatment was 29.6 ± 5.1 ng/mg protein (BCS/DPen), 32.1 ± 5.5 ng/mg protein (5 μM copper) and 33.4 ± 7.2 ng/mg protein (50 μM copper) in the control cells. The levels of iron in the ATP7A overexpressing cells were not significantly different to the controls although there was a trend indicating that there may be slightly more iron in the ATP7A overexpressing line; 38.3± 7.6 ng/mg protein (BCS/DPen), 38.0± 4.9 ng/mg protein (5 μM copper) and 41.2 ± 8.8 ng/mg protein (50 μM copper) ($p>0.05$). No significant variation was detected in iron levels between the various copper treatments for both cell types ($p>0.05$) (Fig 5.10b).

Copper localised predominantly in the central region of wild type PMC42-LA cells treated with 50 μM copper as previously described in chapter 3. Synchrotron X-ray fluorescence was also used to analyse the metals in ATP7A overexpressing cells. Copper localised near the nuclear and perinuclear regions of the ATP7A overexpressing cells treated with 50 μM copper. Copper levels in the 5 μM copper treated cells were on the fringe of

detection like the control cells and so no clear observations of localisation could be made. Copper could not be detected in the BCS/DPen treated cells (Fig 5.11c). Zinc localisation was used to identify the position of cells. Analysis of the images using GeoPIXE software revealed that copper levels within the ATP7A overexpressing cells were slightly reduced compared to the control cells when treated 50 μM copper (ATP7A overexpressing cells 23.8 ppm, control cells 27.8 ppm); consistent with the AAS data (Fig 5.10a). Measurement of iron levels showed little difference between copper treatments or between the two cell lines (Fig 5.11).

Figure 5.10 Intracellular copper and iron levels in PMC42-LA cells in response to ATP7A overexpression

The graphs show average values of copper (a) and iron (b) levels in ATP7A overexpressing PMC42-LA cells treated with BCS/DPen, 5 μM copper and 50 μM copper compared to control PMC42-LA cells. Statistical variation is indicated by different letters above graph bars ($p<0.05$).

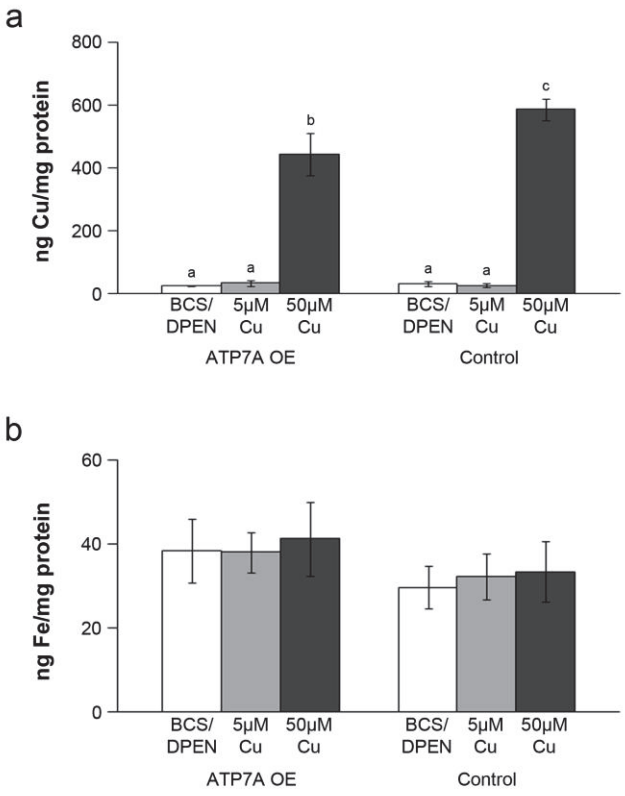
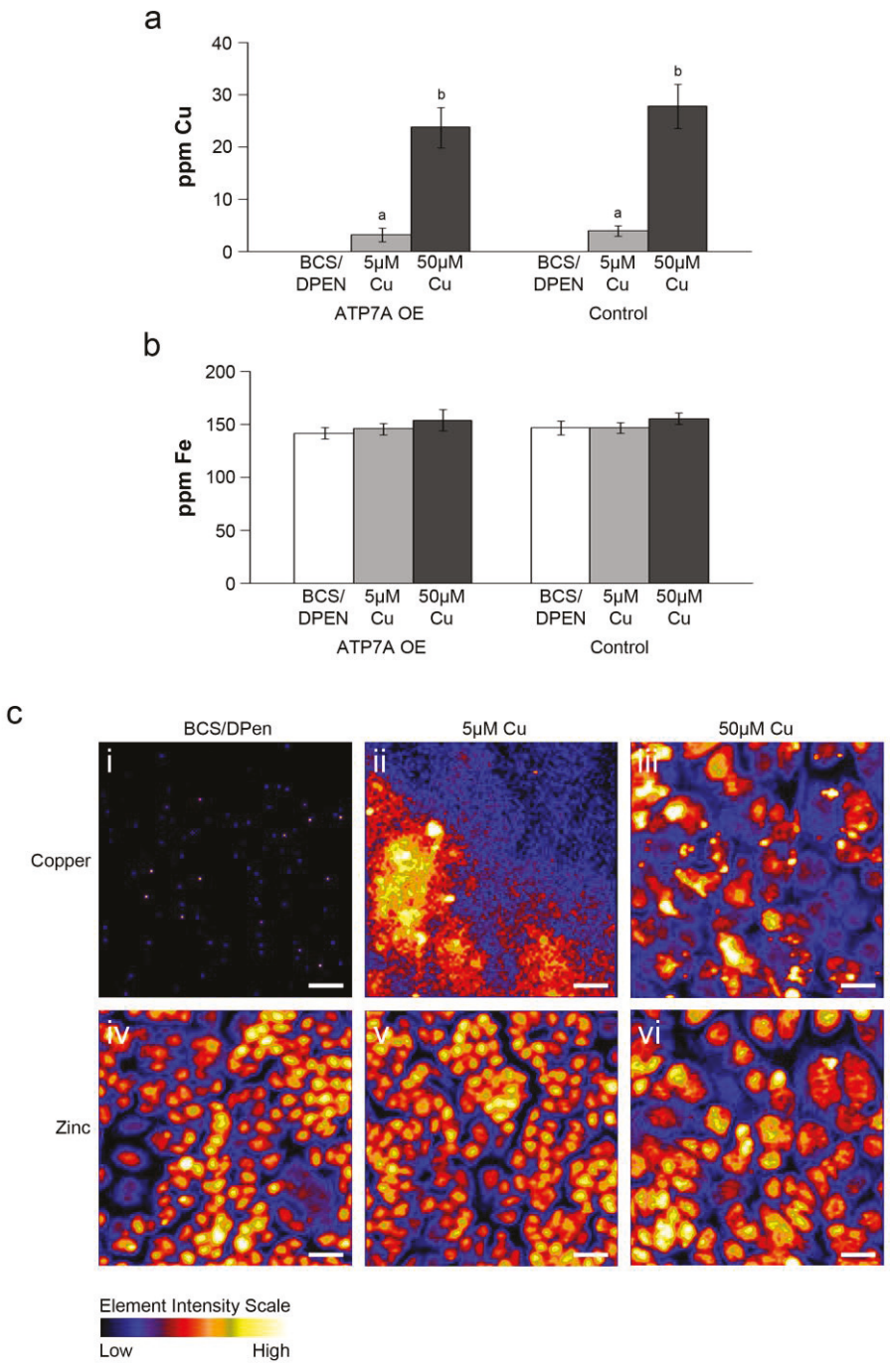

a
ng Cu/mg protein
800
600
400
200
0
a
a
b
a
a
c
BCS/ DPEN
5μM Cu
50μM Cu
BCS/ DPEN
5μM Cu
50μM Cu
ATP7A OE
Control
b
ng Fe/mg protein
60
40
20
0
BCS/ DPEN
5μM Cu
50μM Cu
BCS/ DPEN
5μM Cu
50μM Cu
ATP7A OE
Control

Figure 5.11 Intracellular copper and iron levels and copper and zinc localisation in PMC42-LA cells in response to ATP7A overexpression

Analysis of the total area of each image using GeoPIXE software revealed levels of copper (a) and iron (b) in the overexpressing and control PMC42-LA cells. Statistical variation is indicated by different letters above graph bars ($p<0.05$). (c) XFM analysis showing the distribution of copper and zinc in PMC42-LA cells treated with BCS/DPen (i and iv), 5 μM copper (ii and v) and 50 μM copper (iii and vi). The colour scale bar shows the relative levels of copper from lowest (black) to highest (white). Length scale bar = 30 μm.


a
ppm Cu
40
30
20
10
0
a
b
a
b
BCS/ DPEN
5μM Cu
50μM Cu
BCS/ DPEN
5μM Cu
50μM Cu
ATP7A OE
Control
b
ppm Fe
200
150
100
50
0
BCS/ DPEN
5μM Cu
50μM Cu
BCS/ DPEN
5μM Cu
50μM Cu
ATP7A OE
Control
c
BCS/DPen
5μM Cu
50μM Cu
Copper
Zinc
i
ii
iii
iv
v
vi
Element Intensity Scale
Low
High

5.3 Discussion

The mammary gland is one of the few organs that express ATP7A, ATP7B and ceruloplasmin (Cp) simultaneously (Ackland et al., 1999, Linder et al., 1998, Llanos et al., 2008, Michalczyk et al., 2008, Tsymbalenko et al., 2009, Yang et al., 1990) however the intracellular relationships between these three transporters have not been thoroughly inverstigated. By transfecting the pCMB344 ATP7A overexpression plasmid into the PMC42-LA mammary gland carcinoma cell line, the homoestatic balance of the efflux proteins was disrupted. The impact this had upon copper and iron levels within the cells and the proteins involved in their transport was analysed. In conjunction with this, the effect of various copper conditions in both the transfected and control cells was also analysed to further understand the dynamics of copper homeostasis in mammary epithelial cells.

ATP7A overexpressing plasmids have been previously introduced into CHO cells, the human skin fibroblast cell line Me32a and in the human ovarian carcinoma 2008 cell line (Bellingham et al., 2004, La Fontaine et al., 1998a, Petris and Mercer, 1999, Samimi et al., 2004). PMC42-LA cells overexpressing ATP7A were analysed by qRT-PCR, Western blot and immunofluorescence microscopy to confirm that the plasmid was expressing ATP7A and that the ATP7A protein produced was functional, thus making these cells an effective model for analysis of copper homeostatic disturbance. Analysis of mRNA and protein levels indicated that ATP7A was elevated in comparison to the levels observed in control cells. Additionally, immunolocalisation confirmed that upon treatment with copper, ATP7A

trafficked towards the plasma membrane, away from the TGN where it was observed in the presence of copper chelators. There was no clear difference in ATP7A localisation between control and ATP7A overexpressing cells indicating that the additional protein was trafficking normally and not being retained in the endoplasmic reticulum or TGN. Biotinylation revealed that the ATP7A overexpressing cells had more protein at the plasma membrane compared to controls suggesting that these cells should be able to efflux more copper than the controls. No changes to *ATP7A* mRNA and protein levels in response to copper were identified; consistent with previous studies which suggest that ATP7A mediated copper efflux is regulated by protein trafficking rather than changes in levels (Monty et al., 2005, Petris et al., 1996).

Previous studies show that increased expression of ATP7A reduces cellular copper accumulation by increasing the rate of copper efflux, making cells more resistant to copper toxicity (Bellingham et al., 2004, La Fontaine et al., 1998a, Petris and Mercer, 1999, Samimi et al., 2004). The atomic absorption spectroscopy results revealed that ATP7A overexpression in PMC42-LA cells reduced intracellular copper compared to control cells; however this effect was only observed in cells treated with 50 μM copper which overall had much higher intracellular copper levels than those exposed to the basal copper treated and copper chelated samples as was also demonstrated in chapter 3. This correlated with the biotinylation data which showed a significant increase in ATP7A protein at the plasma membrane in 50 μM copper treated ATP7A overexpressing cells compared to controls. Intracellular copper levels remained at a basal level in either the presence of copper chelators BCS and

DPen, or when exposed to a physiological level of copper (5 μM copper) in both control and ATP7A overexpressing cells, consistent with the experiments of chapter 3 and also previous findings (Hamza et al., 2003, McArdle et al., 1990, Meshitsuka et al., 1987, Steinebach and Wolterbeek, 1994, Urani et al., 2003).

To obtain a greater understanding of how cells respond to high copper loads (50 μM copper), biotinylation was used establish whether either of the two ATPases were localised at the plasma membrane. The results obtained in this study are in agreement with previous research which showed that copper treated cells increased the amount of ATP7A protein at the plasma membrane compared to copper depleted cells, while not altering the total amount of protein in the cells (Nyasae et al., 2006, Pase et al., 2004). This is consistent with the mechanism of ATP7A regulating intracellular copper levels by inducing protein trafficking, rather than altering expression.

Biotinylation analysis revealed that there was no significant difference in ATP7A levels at the plasma membrane between the 5 μM and 50 μM copper treated cells. This result suggests that the efflux mechanism may be saturated, preventing further export of copper from the cells, thus the cells are unable to effectively efflux copper at high concentrations. This is despite the fact that there was more protein trafficking towards the plasma membrane as indicated by the immunofluorescence microscopy which showed a greater amount of protein dispersed throughout the cells in the 50 μM copper treated cells compared to the 5 μM copper treatment, which still had some ATP7A remaining in the TGN. Additionally, the level of holo-Cp

within and secreted from the cells, as shown in chapter 4, does not change between the 5 μM and 50 μM copper treated cells. This again suggests that there is a limit to the rate of copper efflux from the cells. While efflux rates may be limited, the uptake of copper does not appear to suffer the same fate as there is still some CTR1 at the plasma membrane when cells are exposed to high copper. Limited efflux and unlimited uptake may account for the overall increase in the intracellular copper levels observed in both parental and ATP7A overexpressing PMC42-LA cells treated with 50 μM copper.

Metallothionein 1 and 2 (MT) expression is strongly correlated to the copper levels within the ATP7A overexpressing cells, increasing in expression when intracellular copper levels were elevated in the 50 μM copper treated cells, a result consistent with the observations of the control cells in chapter 3. MT has previously been shown to respond to copper supplementation (Armendariz et al., 2004, Coyle et al., 2002, Prohaska and Gybina, 2004, Urani et al., 2003). It is likely that the copper accumulating within the cells has been sequestered by MT, a metallochaperone that is a storage molecule for metal ions like copper, zinc and cadmium, that protects cells from metal ion toxicity (Mehta et al., 2006, Sutherland and Stillman, 2011, Urani et al., 2003). Data showed that MT levels were lower in the 50 μM copper treated ATP7A overexpressing cells compared to controls, further supporting the functionality of the exogenous ATP7A as it is consistent with the ATP7A overexpressing cells having lower copper levels compared to the control cells.

When copper homeostasis is disturbed, the copper uptake and efflux mechanisms should adjust accordingly to maintain balance. In support of this theory, the data presented in chapter 3 showed that while *CTR1* mRNA remained unaltered, increased copper levels caused post-translational degradation and internalisation of the CTR1 protein from the membrane. Degradation and internalisation are important regulatory mechanisms for CTR1 to prevent the uptake of toxic levels of copper as reported in various cell types (Guo et al., 2004a, Guo et al., 2004b, Holzer and Howell, 2006, Holzer et al., 2004a, Liu et al., 2007, Molloy and Kaplan, 2009, Petris et al., 2003, Safaei et al., 2009). The CTR1 responses observed in ATP7A overexpressing cells were no different from those identified previously in wild type PMC42-LA cells. Total and plasma membrane bound CTR1 was greatly reduced in response to copper, most likely as a result of protein degradation, as no changes to the mRNA levels were detected. As demonstrated previously in chapter 3, differences in levels observed between the biotinylation densitometry and the whole cell lysate densitometry suggested some internalisation. This conclusion is drawn from the fact that the difference in total levels of CTR1 between the BCS/DPen and 5 μM copper treated cells was smaller than the difference in levels present at the plasma membrane, indicating that as copper levels rise, CTR1 is internalised from the plasma membrane. CTR1 levels were elevated in the ATP7A overexpressing cells compared to the control cells in each of the three copper treatments, with biotinylation showing more CTR1 at the plasma membrane of the ATP7A overexpressing cells compared to the control for each copper condition. The explanation for this effect may be that the additional ATP7A in the cells is effluxing more copper, and that the cells may

be attempting to balance this efflux through reduction of CTR1 degradation and moving more protein to the membrane to increase copper uptake into the cells. This is supported by the AAS data which showed no difference in levels between the control and ATP7A overexpressing cells in the BCS/DPen and 5 μM copper treated cells. In the 50 μM copper treated cells, the efflux mechanisms may be saturated, limiting the rate of copper efflux. For intracellular copper levels to remain in equilibrium when exposed to high copper loads, most CTR1 protein would need to be degraded or removed from the plasma membrane. This does not occur, thus causing the observed elevation in intracellular copper levels.

ATOX1 is a copper chaperone that delivers copper from CTR1 to the ATPases, ATP7A and ATP7B. Investigation of the levels of ATOX1 mRNA and protein in response to copper supplementation and copper deficiency has received little attention. However the expression levels of *ATOX1* mRNA have been analysed in the livers and placentae of rats fed copper deficient and iron deficient diets. The iron deficient diet resulted in elevated copper levels in the serum, liver and placenta, whereas the copper deficient diet caused reduction in copper levels in the serum and liver. The change in copper levels in the placenta and liver had no impact upon the *ATOX1* mRNA levels (Andersen et al., 2007, Gambling et al., 2004). Consistent with this, no variations in ATOX1 mRNA or protein levels were observed in response to changes in copper concentration or in response to ATP7A overexpression in PMC42-LA cells. ATOX1 is thought to be primarily involved in the movement of copper from CTR1 to the ATPases in the TGN (Banci et al., 2007, Banci et al., 2009, Hussain et al., 2009, Rodriguez-Granillo et al., 2010, Strausak et

al., 2003, van Dongen et al., 2004, Walker et al., 2002, Xiao et al., 2004, Xiao and Wedd, 2002). It also plays a critical role in copper efflux by influencing trafficking of the ATPases; a process that was abolished upon knockout of the *ATOX1* gene (Hamza et al., 2003). This suggests that ATOX1 could become rate limiting for copper efflux if it were not regulated when demand was high. However it was noted in PMC42-LA cells that the *ATOX1* mRNA levels significantly exceeded the levels of transcript of both *ATP7A* and *ATP7B* (data not shown); a results also observed by Lutsenko et al. (2007b) comparing *ATOX1* to *ATP7B* in HepG2 liver cells. If this translates to an excess of ATOX1 protein, it would ensure that ATOX1 does not become a limiting factor in the movement of copper through the cells.

Upon overexpression of ATP7A in PMC42-LA cells, the response of ATP7B was measured. Previous studies have suggested that these ATPases may be interchangeable, where one protein can perform the role of the other (Barnes et al., 2005, La Fontaine et al., 1998a). However the effect of increasing one of the ATPases on the other has not been tested in cultured cells. ATP7A overexpression had no influence upon ATP7B expression as ATP7B mRNA and protein levels remained unchanged between the overexpressing and control cells in the presence and absence of copper. This is consistent with previous studies which suggest that ATP7B-mediated copper efflux is regulated by protein trafficking rather than changes to mRNA and protein levels (Barnes et al., 2009, Schilsky et al., 1998). The addition and depletion of copper elicited small changes in ATP7B localisation in both control and ATP7A overexpressing cells indicating that ATP7B was behaving as previously described (Michalczyk et al., 2008). Similar to ATP7A, ATP7B

resides in the TGN when copper levels are low and disperses towards the plasma membrane when copper levels are elevated. No clear differences in localisation were observed between the ATP7A overexpressing and control cells. It is possible that fluctuations in copper between the control and ATP7A overexpressing cells are too small to initiate changes in ATP7B that could be observable by immunofluorescence microscopy; even in the 50 μM copper treated cells which showed statistically significant changes in intracellular copper levels. Similarly, ATP7A overexpression in mice also failed to elicit an observable change in ATP7B localisation in mammary epithelial cells compared to the cells of control mice (Llanos et al., 2008).

ATP7B protein was not detected at the plasma membrane in copper treated copper chelated, ATP7A overexpressing and control cells, when subject to surface biotinylation. This result is consistent with the notion that ATP7B does not travel to the plasma membrane, rather it delivers copper to vesicles in the vicinity of the membrane which then proceed to efflux copper from the cell following the departure of ATP7B back towards the TGN (Cater et al., 2006, Hung et al., 1997, Schaefer et al., 1999b, Yang et al., 1997). In chapter 4, ATP7B was shown to interact with holo-Cp in mammary epithelial cells. It is likely that ATP7B delivers its copper to Cp in these vesicles for efflux from the cell.

Cp is secreted from mammary epithelial cells and is known to carry up to 85% of the copper secreted into milk (Platonova et al., 2007, Tsymbalenko et al., 2009) and so it is important to understand the effect of copper homeostatic disruption on this protein, as inadequate or excessive supply of

copper into milk would be detrimental to a suckling infant. Interestingly, the current research revealed a dramatic increase in Cp expression in response to ATP7A overexpression. This was detected in both the mRNA and protein levels. This observation was made for both the apo and holo forms of soluble Cp protein that were within and also secreted from the cells, as well as for the glycosylphosphatidylinositol-linked Cp (GPI-Cp) protein detected at the plasma membrane. This increased expression was not likely a result of fluctuating copper levels or changes to copper efflux from the cells, as it was shown in chapter 4 that *Cp* mRNA levels do not change in response to copper treatments. This is supported by other studies which have shown that copper has no impact on the levels of *Cp* mRNA in livers of mice and rats (McArdle et al., 1990, Mostad and Prohaska, 2011). Copper levels only affect the proportion of apo-Cp (non copper bound) to holo-Cp (copper bound) (chapter 4). This was also the case in the ATP7A overexpressing cells, where the addition of copper resulted in a marked increase in the presence of holo-Cp, while apo-Cp was the dominant form present in copper depleted cells. The impact of ATP7A overexpression on Cp transcription and translation had not been anticipated. There has been limited research showing connections between ATP7A and Cp, however it has been reported that in the developing brain upon knockout of ATP7B, ATP7A may have assumed the role of copper delivery to Cp (Barnes et al., 2005). Further to this, macrophages, which do not express ATP7B, were shown to secrete holo-Cp, and the incorporation of copper into Cp was impeded by silencing ATP7A (White et al., 2009a). A unique hexapeptide sequence in ATP7A between transmembrane domain three and four confers its capacity to bind to Cp, indicating the possibility of a direct interaction between the two

proteins (Tsymbalenko et al., 2000). These three studies suggest that ATP7A is a possible source of copper for Cp. While the data presented in chapter 4 did not reveal a direct interaction between ATP7A and Cp this could be due to the presence of a fully functional ATP7B protein in the PMC42 cells. Having said this, it is unlikely that an interaction between ATP7A and Cp in ATP7A overexpressing cells would be the cause of Cp up-regulation, as this would not explain the increase in *Cp* mRNA levels or the increase in apo-Cp levels.

A wide variety of factors are known to influence the transcription of *Cp* including cytokines like IL-6, IL-1β and Interferon γ, and transcription factors like FoxO1, HIF-1 and AP-1. The structure of the 3' untranslated region of *Cp* mRNA, hypoxia, reactive oxygen species/oxidative stress, cancer, pregnancy and preeclampsia also influence *Cp* levels (Das et al., 2007, Guller et al., 2008, Jaeger et al., 1991, Lee et al., 2004, Mazumder and Fox, 1999, Mukhopadhyay et al., 2000, Persichini et al., 2010, Sidhu et al., 2011, Stein et al., 2009, Tapryal et al., 2009, Zibzibadze et al., 2009). Therefore it is possible that the connection between Cp and ATP7A may involve additional factors. At this stage, the literature does not reveal any connection between ATP7A expression and the factors that influence *Cp* mRNA transcription. Perhaps future work may be carried out to focus on the *Cp* promoter using luciferase constructs to determine the region that is involved in *Cp* up-regulation in the presence of additional ATP7A. This may then shed more light on the pathways involved.

The effect of iron levels on the transcription of *Cp* has yielded contradicting results. Some researchers have observed changes in Cp expression levels (Balusikova et al., 2009, Mukhopadhyay et al., 1998, Qian et al., 2007), while others have reported no change (Balusikova et al., 2009, Chang et al., 2007, Chen et al., 2006, Chen et al., 2009, Fosset et al., 2009, Gambling et al., 2004). The observed changes to the protein levels of β2-microglobulin (β2M) instigated our interest in the effect of iron upon Cp expression despite these conflicting views. β2M protein was significantly elevated in the ATP7A overexpressing cells. β2M protein stability is affected by the presence of Cu (II) (Deng et al., 2006) and so it is likely that the additional ATP7A is reducing the pool of copper that is available for destabilising β2M and preventing its degradation rather than influencing transcription levels which were unaffected. Interestingly, β2M plays an important role in binding to HFE, a protein which impedes the binding of transferrin receptor 1 (TfR) to iron loaded plasma transferrin and inhibits iron uptake into the cell (Bhatt et al., 2009, Bhatt et al., 2010, Enns, 2001, Vecchi et al., 2010). An increase in TfR did not accompany the rise in β2M levels, an effect also seen in CHO cells overexpressing β2M (Wang et al., 2003). However the increased β2M could be binding more HFE, preventing the HFE-TfR complex from being created, thus increasing the amount of TfR available to interact with transferrin which would enable increased uptake of iron. It has been shown previously that CHO cells overexpressing β2M increase their iron uptake (Waheed et al., 2002). A slight increase in intracellular iron levels was observed in the ATP7A overexpressing cells. While the increase in iron was not significant, this does not discount that an increase in efflux could be balancing increased uptake, similar to the observations of copper in ATP7A overexpressing cells

at basal levels. If iron uptake stimulated Cp transcription, Cp could initiate the efflux of iron from the cells. However this would not be the case in the copper deficient cells as there is no ferroxidase active holo-Cp in these cells which is required to mobilise iron. Further to this, incubation of PMC42-LA cells in iron had no impact upon the levels of Cp and so it is unlikely that iron played a role in elevating Cp transcription in ATP7A overexpressing cells.

Some recent work has identified that β2M has a role in cellular proliferation and is up-regulated in some cancers (Huang et al., 2008, Huang et al., 2006, Josson et al., 2011, Rowley et al., 1995, Yang et al., 2006). ATP7A and Cp have also shown to be up-regulated in tumours (Chen et al., 2012, Nagaraja et al., 2006, Owatari et al., 2007, Samimi et al., 2003, Stein et al., 2009, Tye et al., 2008, Woo et al., 2009, Youn et al., 2009), while Cp was also reported to be elevated in the blood when tumours are present (Cox et al., 2001, Geetha et al., 2009, Tye et al., 2008, Zibzibadze et al., 2009). It is possible that the overexpression of ATP7A in the PMC42-LA cells has activated some linking factors that connect the three proteins. ATP7A may also be initiating signalling pathways that are yet to be elucidated. Research has shown that changes in ATP7A protein levels have been linked with regulating the expression of VEGFR1 and Prnp, independently of a copper effect (Afton et al., 2009, Armendariz et al., 2004), similar to the results observed for Cp.

One possible pathway that links ATP7A to Cp could be via MAPK/ERK. MAPK/ERK, a pathway involved in cellular growth and proliferation is activated by β2M (Huang et al., 2010, Mattie et al., 2008, Yang et al., 2006). *Cp* has activator protein-1 (AP-1) binding sites, and the removal of these

sites impedes transcription of *Cp* (Das et al., 2007, Lee et al., 2004, Mukhopadhyay et al., 2000, Tapryal et al., 2010, Tsymbalenko et al., 2009). Interestingly, it has been shown that AP-1 is controlled by the MAP kinases including ERK (Karin, 1996) and so it is possible that the increase in β2M is instigating the changes in Cp expression.

The *Cp* mRNA results presented here contradict those presented by Llanos et al., (2008) in which a mouse model overexpressing ATP7A showed no significant difference in Cp mRNA or protein levels. These mice were overexpressing human ATP7A and so a logical explanation is that the human protein, while capable of trafficking copper and influencing the copper secretion into milk, may be different enough from the mouse ATP7A (Qian et al., 1998) to be incapable of influencing the Cp mRNA and protein levels as found in the human PMC42-LA cell line.

The large increase observed in Cp levels in ATP7A overexpressing cells did not correspond to any changes in its localisation within the cells. Cp appeared granular throughout the cells, similar to the observations made of wild type PMC42-LA cells in chapter 4.

5.4 Conclusion

ATP7A is an important component of the cellular copper efflux pathway. Overexpressing ATP7A affected the copper uptake protein CTR1, intracellular copper levels and copper storage protein MT. In 50 μM copper treated cells ATP7A overexpression significantly decreased the level of intracellular copper. Intracellular copper levels corresponded with the MT protein levels. Less MT was detected in the overexpressing cells when it was expressed in the 50 μM copper treated cells, suggesting that there was less copper that required storing. CTR1 protein levels increased in response to ATP7A overexpression, a response which may accommodate an increased rate of efflux caused by ATP7A. This would be particularly important in cells treated with copper chelators and those having basal copper levels as elevated efflux would leave the cells copper deficient. Interestingly ATP7A overexpression influenced iron transport pathways, causing increases in β2M and Cp. While the change in β2M is likely copper related, the change in Cp is perplexing and perhaps there are signalling mechanisms that ATP7A instigates that are yet to be identified. This research has given insight into the copper homeostatic mechanisms in a system containing three copper efflux proteins and has also revealed some interesting results which opens the door for more in depth analysis and the possibility of identifying new roles for ATP7A.

CHAPTER 6

Copper homeostasis is altered by ATP7A overexpression in differentiated PMC42-LA mammary epithelial cells

6.1 Introduction

ATP7A is a copper transporting protein involved in removing excess copper from cells, preventing the accumulation of toxic copper levels. The overexpression of ATP7A in undifferentiated PMC42-LA cells influenced proteins involved in maintaining copper and iron homeostasis. Ceruloplasmin (Cp) and β2-microglobulin (β2M) levels increased, while CTR1 and metallothionein 1 and 2 (MT) levels decreased in the presence of additional ATP7A protein. The changes in β2M, CTR1 and MT were likely due to increased efflux of copper which also caused a decrease in copper accumulation in high copper treated cells.

In the physiological conditions of the mammary epithelium, ATP7A's role in copper efflux may be more important than in other cell types. Localising basolaterally, ATP7A is hypothesised to increase the return of copper from the mammary epithelium back into the maternal bloodstream, thereby impeding the movement of copper into the milk and so in addition to maintaining cellular copper levels it may also be protecting a suckling infant from exposure to toxic copper levels (Llanos et al., 2008).

The aim of this research was to investigate copper homeostasis in mammary epithelial cells; a unique system which contains three copper efflux molecules. Continuing on from the undifferentiated PMC42-LA cells analysis, this current chapter describes the responses associated with differentiated PMC42-LA cells overexpressing ATP7A. As described in chapter 3 and 4, differentiated cultures were treated with lactational hormones to mimic

lactation and the suckling response. This enabled analysis of homeostatic disruption in a system more representative of *in vivo* conditions.

A number of results were similar to those observed throughout chapters 3-5, however some variations and novel responses were observed including hormonally induced trafficking of ATP7A and copper induced changes in ATP7B expression. The data obtained showed that the mammary epithelial cells utilise a number of mechanisms in the presence of homeostatic disturbances of copper that ensure an adequate flux of copper across the apical membrane and enabling a suckling infant to be properly nourished.

6.2 Results

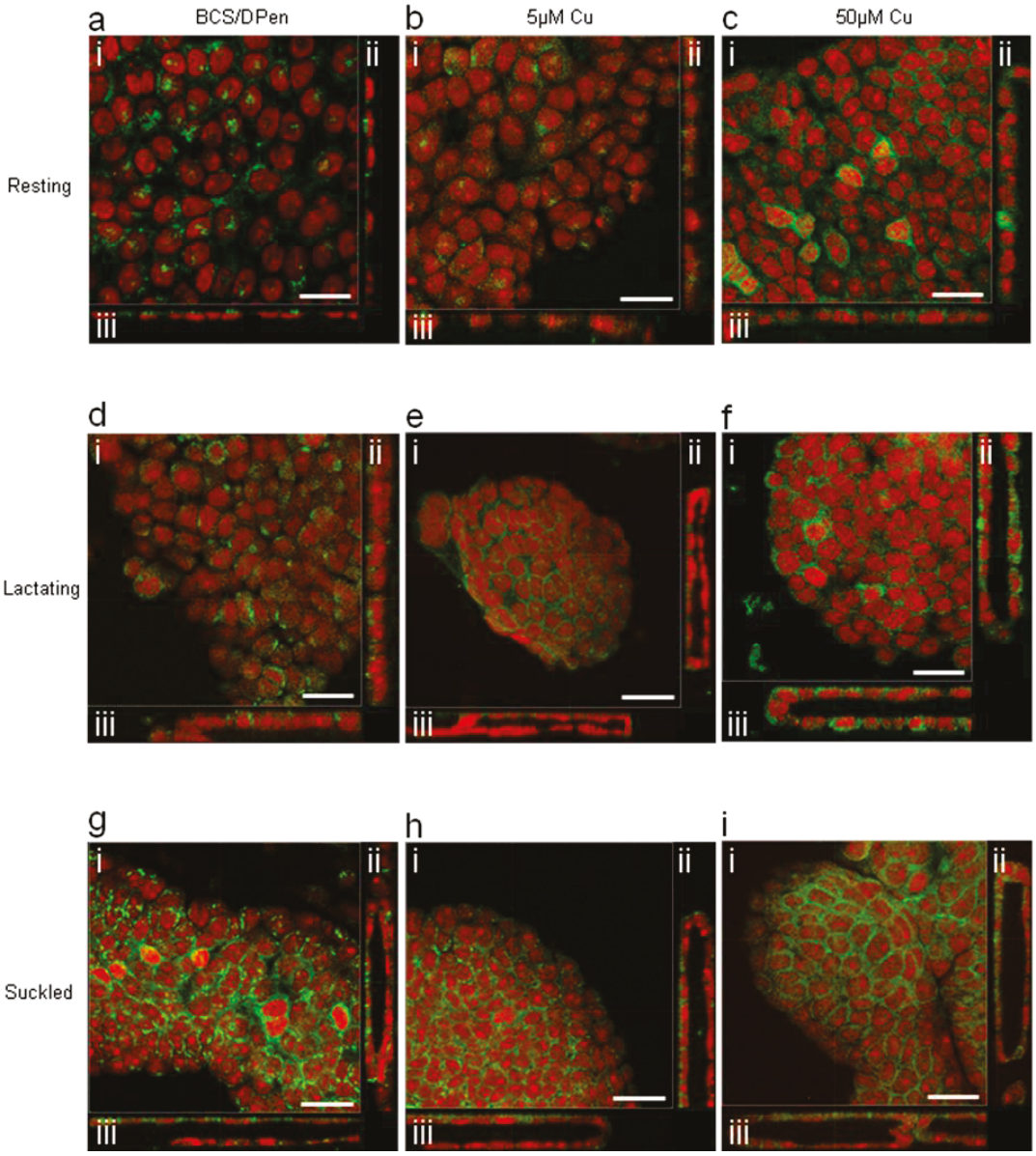
6.2.1.1 Copper induces trafficking of ATP7A, but does not affect the expression of ATP7A mRNA or protein in differentiated PMC42-LA cells

ATP7A has been shown previously to be more dispersed in lactating human mammary epithelial cells compared to non-lactating cells (Ackland et al., 1999). Immunocytochemistry and confocal microscopy were used to determine whether the distribution of ATP7A in the resting, lactating and suckled differentiated culture models was consistent with the localisation in tissues. Confocal microscopy enabled protein localisation within three axes of individual cells and entire organoids to be determined. The EHS matrix surface on which the organoids were grown, is located on the right hand side of image ii and the lower side of image iii.

Depleting the resting breast model cells of copper resulted in tight perinuclear labelling of ATP7A consistent with a TGN localisation (Fig 6.1a). As copper concentrations increased, ATP7A became more dispersed, showing a granular cytoplasmic distribution with some perinuclear labelling in cells treated with 5 μM copper (Fig 6.1b). Further dispersion of ATP7A into the cytoplasm occurred in cells treated with 50 μM copper (Fig 6.1c). No perinuclear labelling could be seen with the 50 μM treatment. This pattern of distribution was observed in all three cell models (Fig 6.1d-i) of differentiated cells. In all models, ATP7A was localised towards the basolateral side of the organoids' cells.

Figure 6.1 Localisation of ATP7A in differentiated PMC42-LA cells

Localisation of ATP7A (green) in the resting (a, b and c), lactating (d, e and f) and suckled (g, h and i) breast cell models in response to BCS/DPen (a, d and g), 5 μM copper (b, e and h) and 50 μM copper (c, f and i). Image i of each sub-figure is a transverse section through the top of the organoid. Image ii and iii show y-z and x-z sagittal sections respectively, cutting through the centre of the organoid. Nuclei were stained with ethidium bromide (red). Scale bar = 20 μm.


a
BCS/DPen
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5μM Cu
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50μM Cu
Resting
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Lactating
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iii

ATP7A mRNA and protein was analysed by qRT-PCR and Western blot respectively to determine expression levels in response to various copper loads. These analyses revealed that copper treatments had no significant effect upon the amount of ATP7A mRNA or protein in differentiated PMC42-LA cells ($p>0.05$) (Fig 6.2a and b).

Biotinylation of PMC42-LA cells was performed to determine the amount of ATP7A present at the plasma membrane. The data revealed that there was significantly more ATP7A at the plasma membrane in copper treated cells compared to BCS/DPen treated cells ($p<0.05$), while no difference was observed between the 5 μM and 50 μM copper treated samples ($p>0.05$). This pattern was reflected across all three models (Fig 6.2c).

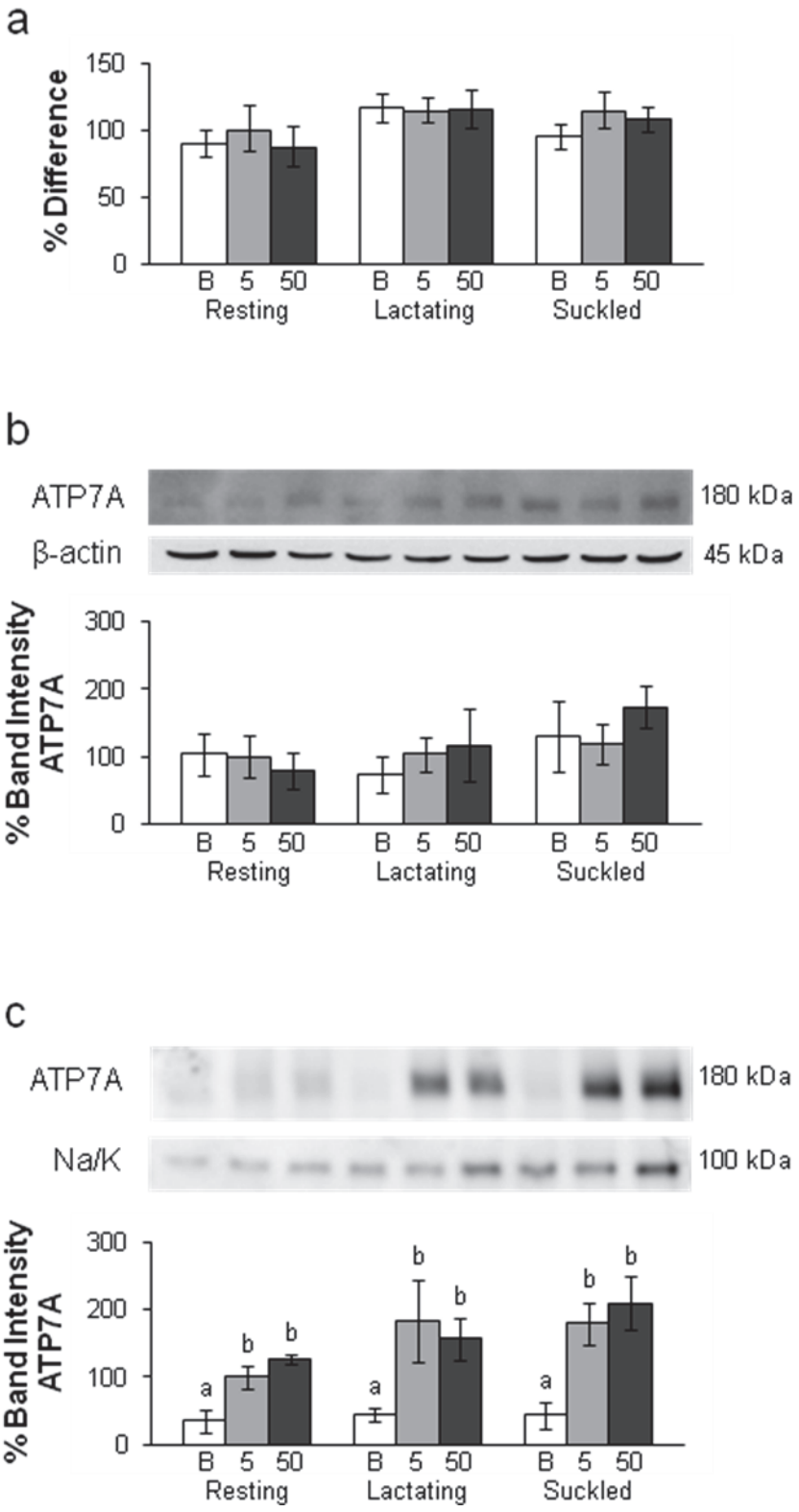
6.2.1.2 *ATP7B* mRNA levels are reduced in response to copper

QRT-PCR revealed that *ATP7B* mRNA was influenced by copper levels in differentiated cells. The addition of 50 μM copper caused *ATP7B* to be significantly down regulated compared to the 5 μM copper treated and copper chelated mRNA levels in the lactating model ($p<0.05$). No statistically significant difference was observed between any of the copper treatments in the resting and suckled models of the control cells ($p>0.05$) (Fig 6.3a).

A band of approximately 160 kDa was detected by Western blot analysis for ATP7B which is consistent for the full length ATP7B protein. The results obtained for ATP7B were extremely inconsistent, therefore three of the five performed experiments are displayed, each from a separate set of lysates

Figure 6.2 ATP7A mRNA and protein expression in differentiated PMC42-LA cells

(a) Relative mRNA levels of *ATP7A* in response to copper and hormones obtained by qRT-PCR. The bar graph indicates the mean percentage differences ±SD compared to the 5 μM copper treated cells of the resting model. ATP7A mRNA levels were normalised against *β-actin*. B = BCS/DPen, 5 = 5 μM copper, 50 = 50 μM copper. Representative Western blots of 60μg of total protein extract (b) as well as surface biotinylated protein (c) from differentiated PMC42-LA cells show ATP7A bands in the resting breast model (lanes 1-3), lactating model (lanes 4-6) and suckled model (lanes 7-9) treated with BCS/DPen (lanes 1, 4 and 7), 5 μM copper (lanes 2, 5 and 8) and 50 μM copper (lanes 3, 6 and 9). β-actin and Na/K ATPase blots demonstrate relative loading of samples. ATP7A densitometry graph bars show mean intensity of ATP7A bands expressed as a percentage of arbitrary units ±SD compared to the 5 μM copper treatment of the resting model, normalised against β-actin or Na/K ATPase for total protein and surface biotinylation respectively. These blots are representative of three independent experiments. Statistical variation is indicated by different letters above graph bars ($p<0.05$).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(Fig 6.3b). Images of Ponceau S stained membranes show even loading for each experiment. The lysates utilised for ATP7B analysis were also used for detection of the other copper transporters, however these did not show the same degree of inconsistency. Obtaining a clear indication of ATP7B's response to copper was difficult due to the highly variable results. When compiling the densitometric analysis of all five blots, a slight down-regulation in response to elevated copper could be identified in some of the models, however due to the large error bars, the results cannot be deemed significant. A second, custom made, ATP7B antibody was also used on control cells lysates and again detection was difficult and no clear outcome could be obtained (data not shown). In spite of the inconsistency, it is possible that ATP7B protein levels may be reduced in response to copper in these differentiated cells, a result which would be consistent with the mRNA data.

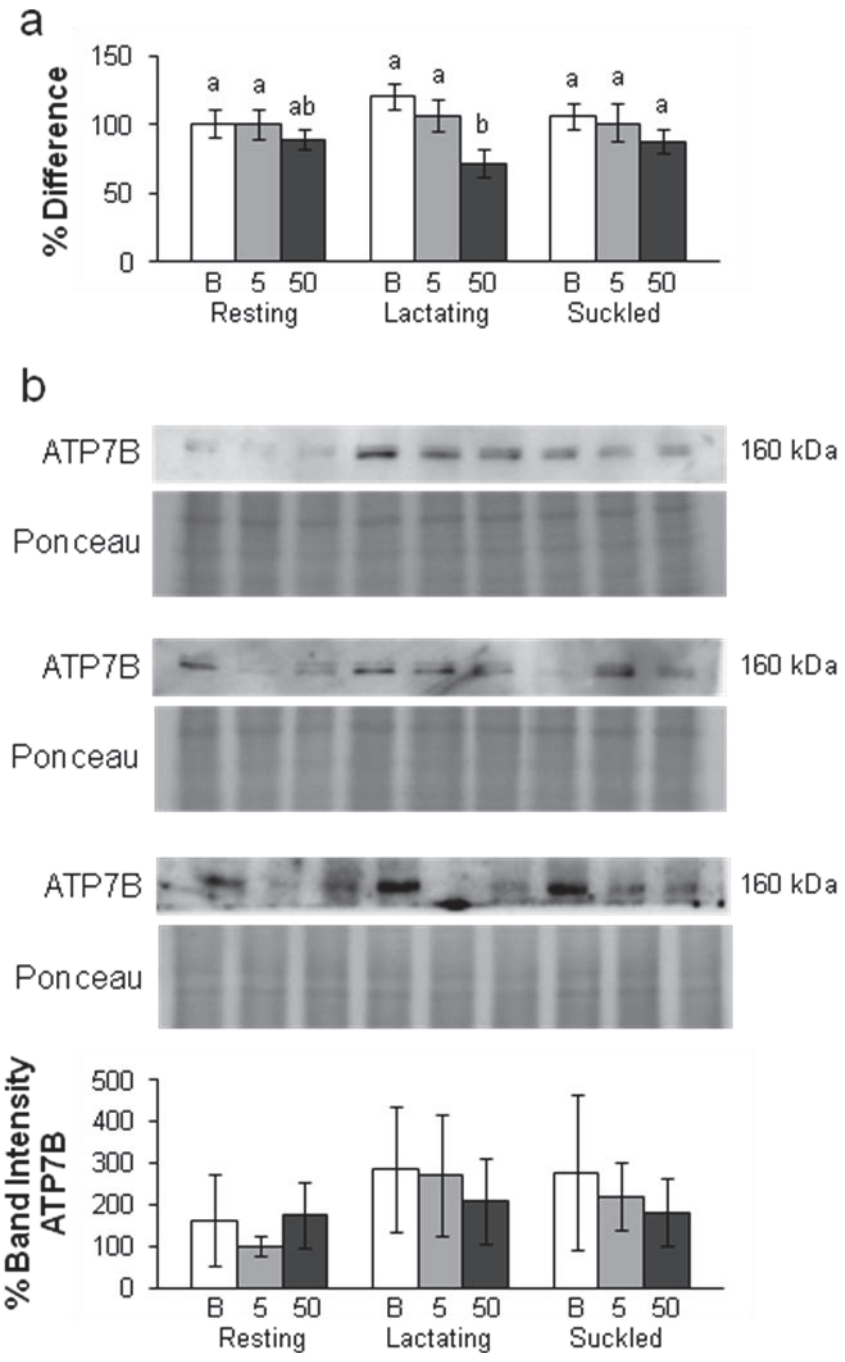
6.2.2.1 Lactational hormones increased the dispersion of ATP7A, but had little effect on expression in differentiated PMC42-LA cells

The addition of lactational hormones caused a slight increase to both the mRNA and protein levels of ATP7A, however these changes were not significant ($p>0.05$). (Fig 6.2a and b).

Surface biotinylation analysis of ATP7A revealed that addition of lactational hormones caused an increase in protein at the plasma membrane for each of the copper treatments, however, similar to total cellular ATP7A levels, this increase was not significant ($p>0.05$) (Fig 6.2c). Further to this, immunofluorescent confocal microscopy analysis of ATP7A revealed that

Figure 6.3 ATP7B mRNA and protein expression in differentiated PMC42-LA cells

(a) Relative mRNA levels of *ATP7B* in response to copper and hormones obtained by qRT-PCR. The bar graph indicates the mean percentage differences ±SD compared to the 5 μM copper treated cells of the resting model. ATP7A mRNA levels were normalised against *β-actin*. B = BCS/DPen, 5 = 5 μM copper, 50 = 50 μM copper. (b) Western blots depicting 60μg of protein extract from differentiated PMC42-LA cells show ATP7B bands in the resting breast model (lanes 1-3), lactating model (lanes 4-6) and suckled model (lanes 7-9) treated with BCS/DPen (lanes 1, 4 and 7), 5 μM copper (lanes 2, 5 and 8) and 50 μM copper (lanes 3, 6 and 9). The Ponceau image demonstrates the relative loading of samples in each replicate. The ATP7B densitometry graph bars show mean intensity of the ATP7B bands from five independent experiments expressed as a percentage of arbitrary units ±SD compared to the 5 μM copper treatment of the resting model, normalised against Ponceau. Blots show three of the five experiments performed.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exposure to lactational hormones caused ATP7A to disperse. This was evident in the presence of copper and also copper chelators. The addition of extra prolactin in the suckled model further enhanced the dispersion of ATP7A (Fig 6.1).

6.2.2.2 Lactational hormones had no impact upon ATP7B mRNA or protein levels

The addition of lactational hormones had no influence upon *ATP7B* mRNA in differentiated cells ($p>0.05$) (Fig 6.3a). A similar trend was observed in the protein. Densitometric analysis indicated that hormones likely had no influence upon ATP7B protein levels, however the blotting was inconsistent as stated earlier (Fig 6.3b).

6.2.3.1 Localisation of ATP7A in the overexpressing system does not change in comparison to endogenous ATP7A localisation

ATP7A was overexpressed in differentiated PMC42-LA cells to determine the effect of this homeostatic distubance upon copper transporting proteins in a system reminiscent of the physiological conditions of mammary epithelial cells. Increases in ATP7A expression *in vivo* could ultimately lead to copper deficient milk and provide inadequate nourishment to a suckling infant if the imbalance is not corrected by other proteins.

The ATP7A localisation observed in these ATP7A overexpressing cells (Fig 6.4) did not alter from the pattern observed in control cells (Fig 6.1) only the staining intensity was slightly greater. Surface biotinylation suggested there was slightly more ATP7A at the plasma membrane compared to control cells

in each respective treatment, however this change was not statistically significant ($p>0.05$) (Fig 6.5a, b and c).

6.2.3.2 *ATP7B* mRNA levels are influenced copper and hormones in ATP7A overexpressing PMC42-LA cells

ATP7A overexpression had no influence upon *ATP7B* mRNA expression in the resting model ($p>0.05$), however in combination with lactational hormones, levels were significantly higher than in the control cells ($p<0.05$) (Fig 6.6a). ATP7B levels were also significantly altered by copper in ATP7A overexpressing cells. There was significantly less ATP7B mRNA in 50 μM copper treated cells compared to the 5 μM copper treated and copper chelated cells in the resting, lactating and suckled models ($p<0.05$) (Fig 6.6a). This data is consistent with the trend observed in the parental differentiated PMC42-LA cells. These trends were not reflected in protein levels due to inconsistencies in blotting with the ATP7B antibody (Fig 6.6b-d).

6.2.3.3 *Cp* mRNA as well as sCp and GPI-Cp protein levels increased in response to ATP7A overexpression

ATP7A overexpression caused a significant increase in total *Cp* mRNA levels compared to the control for each individual treatment ($p<0.05$) (Fig 6.7a). Protein levels also followed this trend. Apo and holo-Cp detected within and also secreted from ATP7A overexpressing cells, strongly exceeded the levels of the control cells ($p<0.05$) (Fig 6.7b-g). The levels of Glycosylphosphatidylinositol-linked Cp (GPI-Cp) detected at the plasma membrane by surface biotinylation was also increased significantly in response to ATP7A overexpression ($p<0.05$) (Fig 6.7h-j).

Figure 6.4 ATP7A localisation in differentiated ATP7A overexpressing PMC42-LA cells

Localisation of ATP7A (green) in the resting (a, b and c), lactating (d, e and f) and suckled (g, h and i) breast cell culture models in response to BCS/DPen (a, d and g), 5 μM copper (b, e and h) and 50 μM copper (c, f and i). Image i of each sub-figure is a transverse section through the top of the organoid. Image ii and iii show y-z and x-z sagittal sections respectively, cutting through the centre of the organoid. Nuclei were stained with ethidium bromide (red). Scale bar = 20 μm.

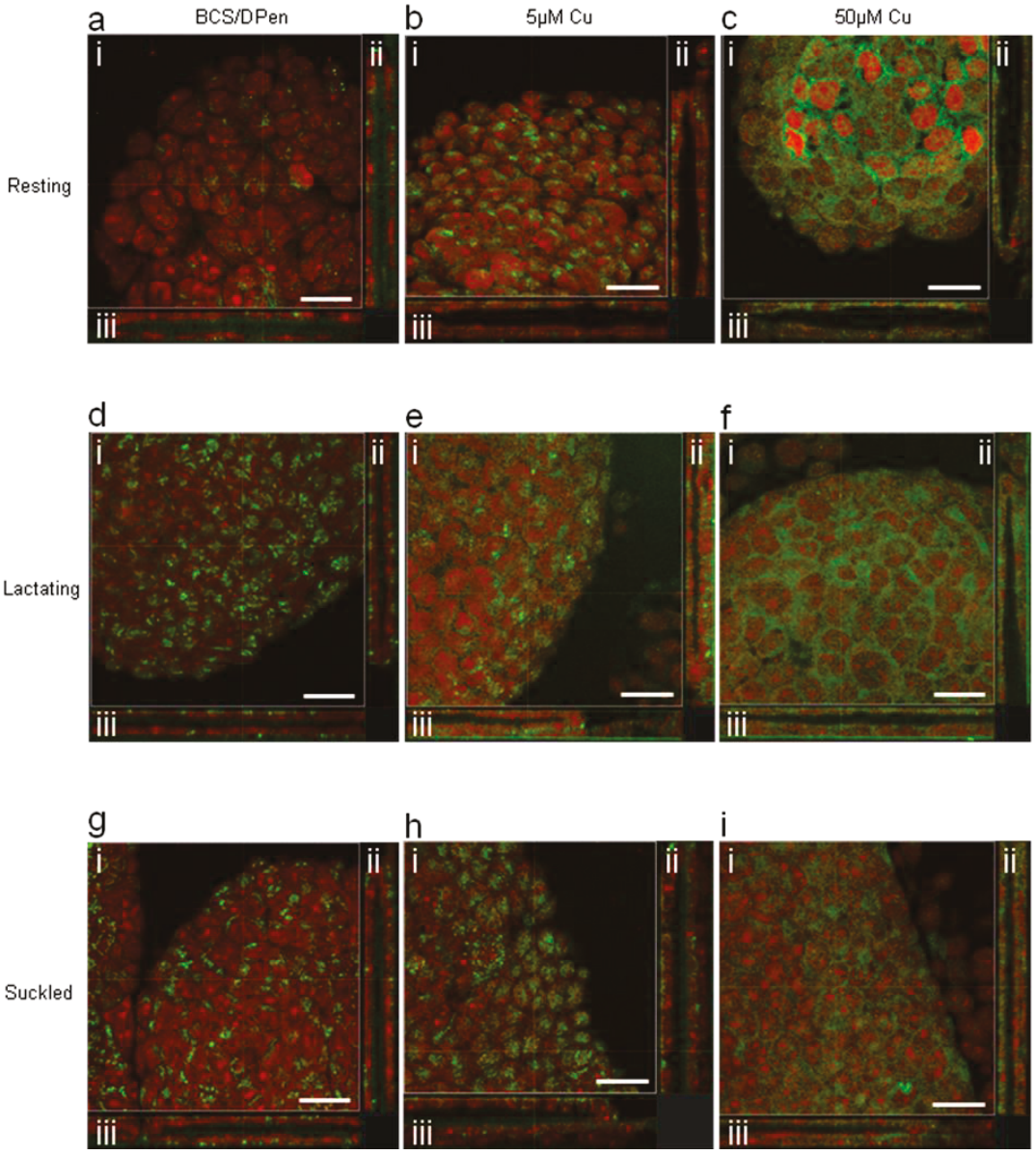
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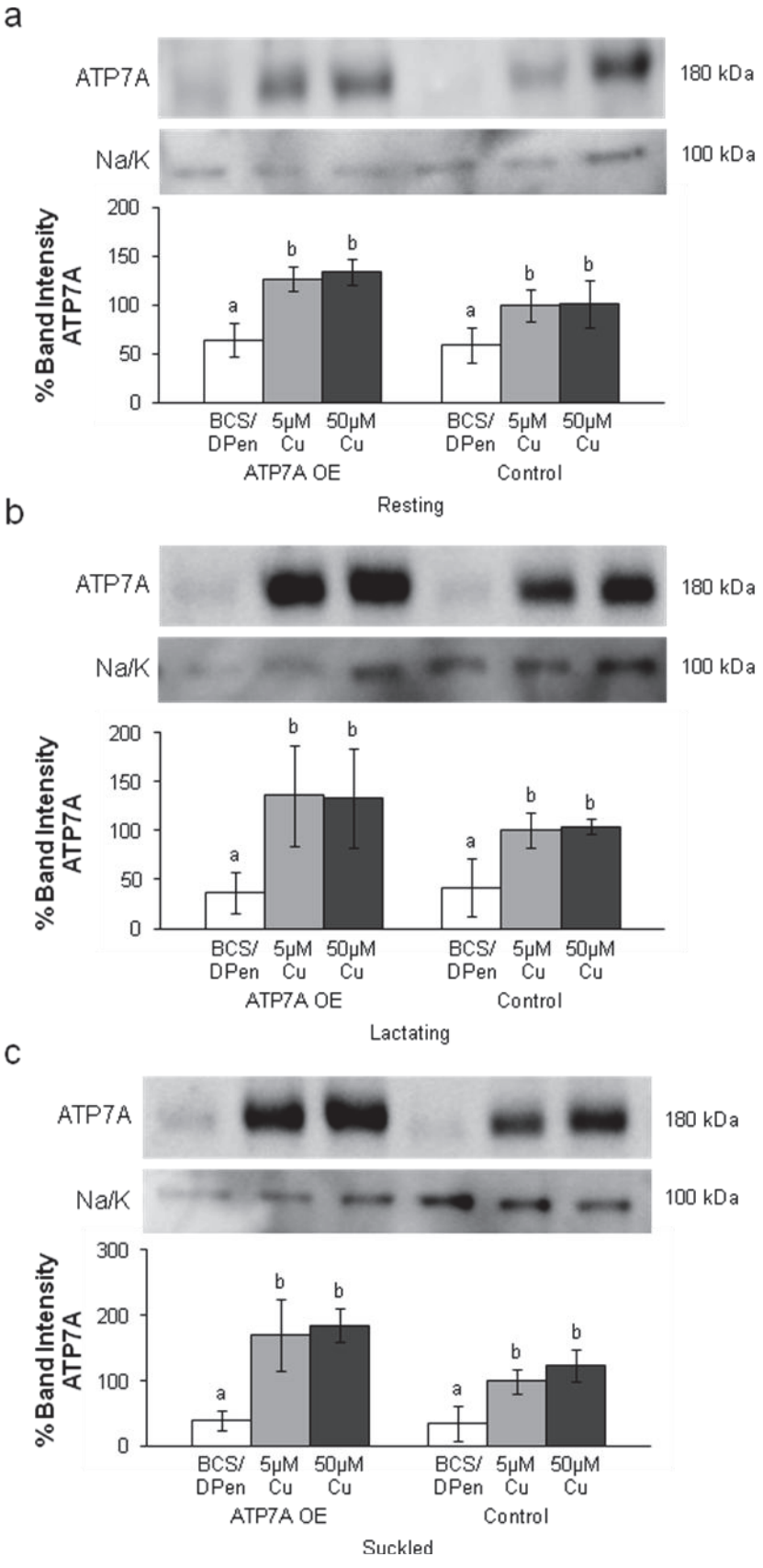
Figure 6.5 Plasma membrane levels of ATP7A in copper and hormone treated PMC42-LA cells

Surface biotinylated ATP7A protein was analysed by Western blot. These representative Western blots of extracellularly biotinylated protein extract show the ATP7A band in ATP7A overexpressing and control cells of the resting breast model, lactating model and suckled model treated with BCS/DPen, 5 μM copper and 50 μM copper. Densitometric analysis showed the mean intensity of ATP7A bands expressed as a percentage of arbitrary units ±SD compared to the 5 μM copper treatment of the control cells, normalised against the Na/K ATPase. The data represents four independent experiments. Statistical variation is indicated by different letters above graph bars ($p<0.05$).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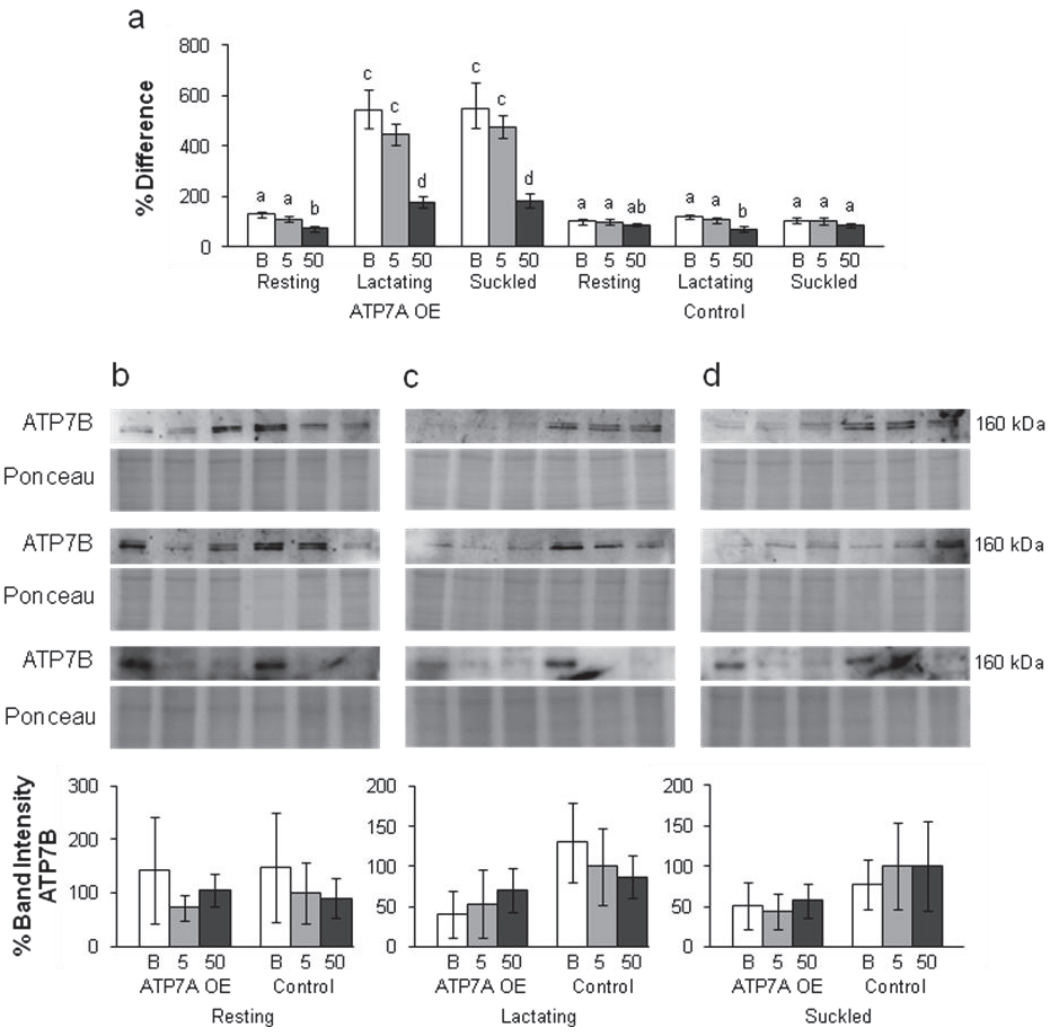
BCS/ DPen
5μM Cu
50μM Cu
ATP7A OE
Control
Suckled

Figure 6.6 ATP7B mRNA and protein expression levels in response to copper, hormones and ATP7A overexpression

(a) Relative mRNA levels of *ATP7B* in response to copper and hormones obtained by qRT-PCR. The bar graph indicates the mean percentage differences ±SD compared to the 5 μM copper treated cells of the control resting model. ATP7B mRNA levels were normalised against *β-actin*. B = BCS/DPen, 5 = 5 μM copper, 50 = 50 μM copper. Western blots show 60μg of protein extract from ATP7A overexpressing and control cells in each of the resting (b), lactating (c) and suckled (d) models treated with BCS/DPen, 5 μM copper and 50 μM copper. The Ponceau images demonstrate relative loading of samples in each replicate. ATP7B densitometry graph bars show mean intensity of ATP7B bands expressed as a percentage of arbitrary units ±SD compared to the 5 μM copper treatment of the control cells, normalised against Ponceau. Blots represent three of the five experiments performed.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Suckled

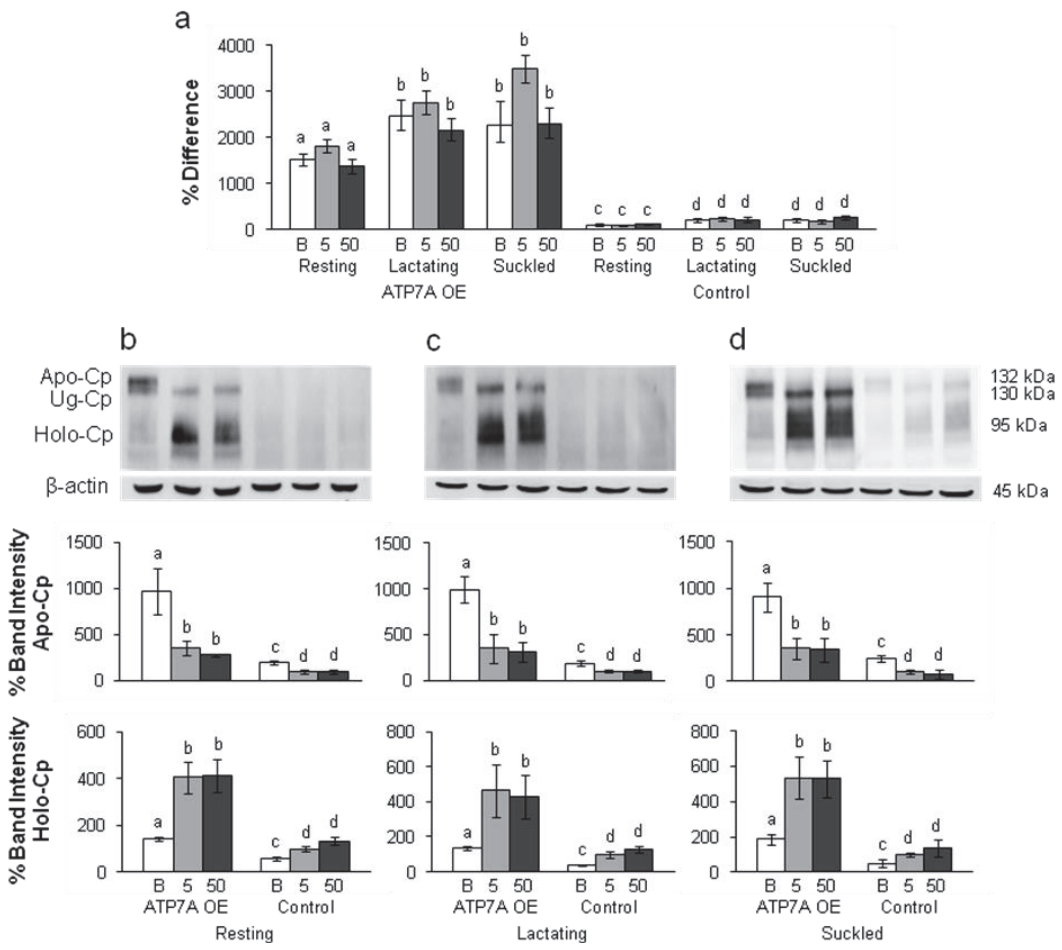
Cp mRNA was unaltered by copper treatments, but increased significantly in response to lactational hormones in ATP7A overexpressing cells (Fig 6.7a). This data reflected the results obtained for the parental cells presented in chapter 4. The copper and lactational hormone responses of Cp protein in ATP7A overexpressing cells also reflected the results obtained in chapter 4. Copper increased the level of holo-Cp present within and secreted from the ATP7A overexpressing PMC42-LA cells, while in copper chelated cells, apo-Cp was the predominant form (6.7b-g). The addition of lactational hormones had no influence upon the total Cp protein within the cells, however induced an increase in protein secretion from the cells. GPI-Cp detected at the plasma membrane of overexpressing cells was not altered by the copper or hormonal treatments. No variation in Cp localisation was observed by immunofluorescent confocal microscopy (data not shown).

6.2.3.4 CTR1 mRNA and protein increased in the presence of additional ATP7A

Overexpression of ATP7A had no effect upon the level of *CTR1* mRNA in the resting model, however, when lactational hormones were present, *CTR1* mRNA levels were significantly elevated compared to control cells ($p<0.05$) (Fig 6.8a). Contrary to this, CTR1 protein levels in ATP7A overexpressing cells were elevated in all three models. Significant differences were detected between the control and ATP7A overexpressing cells treated with BCS/DPen and 5 μM copper ($p<0.05$), however no differences were observed between the 50 μM copper treated cells ($p>0.05$) (Fig 6.8b). Plasma membrane bound CTR1 protein levels reflected total CTR1 protein, with all samples having more protein at the membrane in ATP7A overexpressing cells compared to

Figure 6.7 Cp mRNA and protein in differentiated PMC42-LA cells

(a) Relative mRNA levels of *Cp* in response to ATP7A overexpression, copper and hormones obtained by qRT-PCR. The bar graph indicates the mean percentage differences ±SD compared to the 5 μM copper treated cells of the control resting model. *Cp* mRNA levels were normalised against *β-actin*. B = BCS/DPen, 5 = 5 μM copper, 50 = 50 μM copper. Representative Western blots of 60μg of protein, comparing the levels of apo-Cp and holo-Cp in whole cell extracts from the resting (b), lactating (c) and suckled (d) models as well as the levels of apo and holo-Cp secreted from these cells (e, f and g) in ATP7A overexpressing cells to control cells. The β-actin blots (lysates) and Ponceau images (secretions) demonstrate relative loading of samples. Cp densitometry graph bars show mean intensity of Cp bands expressed as a percentage of arbitrary units ±SD compared to the 5 μM copper treatment of control cells, normalised against β-actin (lysates) or Ponceau (secretions) and is representative of three independent experiments. Statistical variation is indicated by different letters above graph bars ($p<0.05$). Surface biotinylated GPI-Cp protein was analysed by Western blot. These representative Western blots of extracellularly biotinylated protein extract show the GPI-Cp band in ATP7A overexpressing and control cells of the resting breast model (h), lactating model (i) and suckled model (j) treated with BCS/DPen, 5 μM copper and 50 μM copper. Densitometric analysis showed mean intensity of GPI-Cp bands expressed as a percentage of arbitrary units ±SD compared to the 5 μM copper treatment of the control cells, normalised against the Na/K ATPase representative of four independent experiments. Statistical variation is indicated by different letters above graph bars ($p<0.05$).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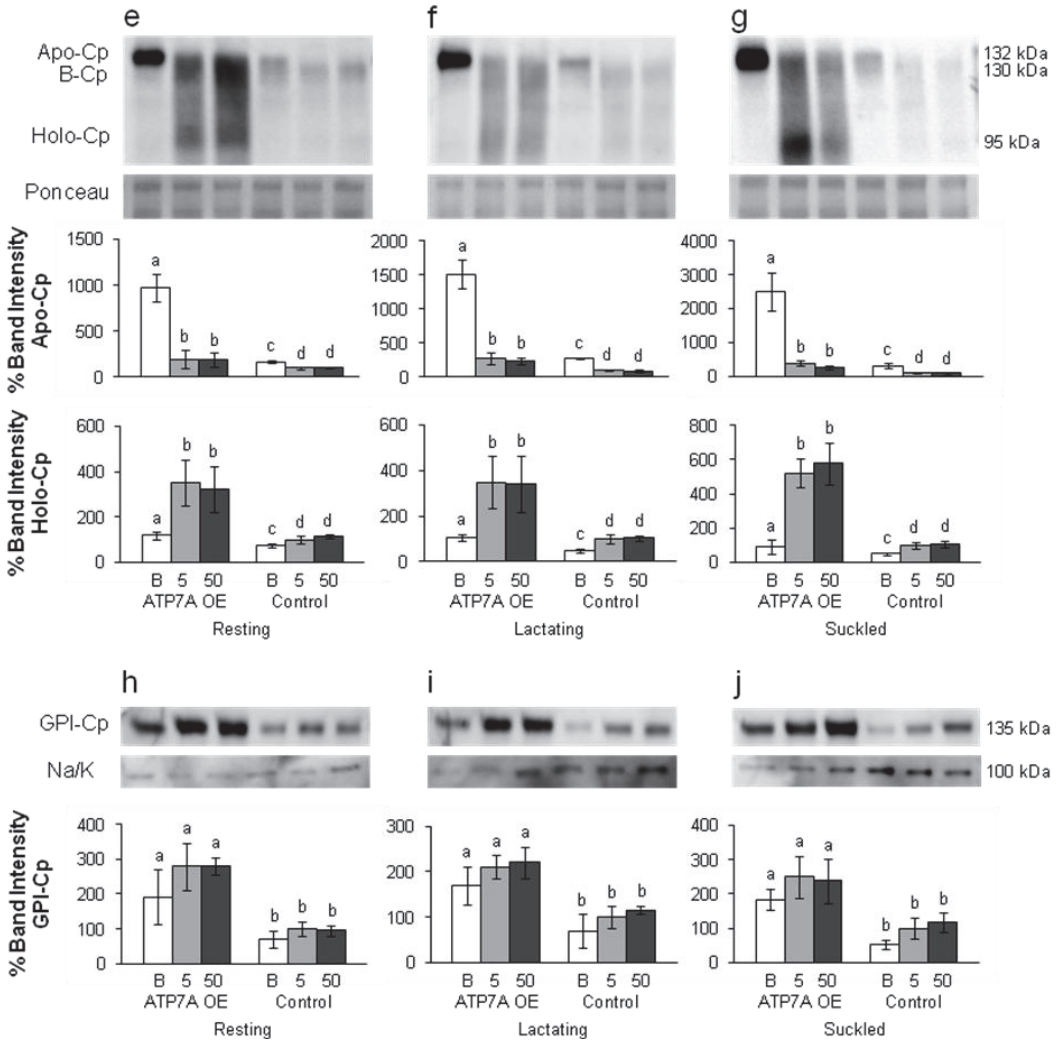
% Band Intensity Holo-Cp
Resting
Lactating
Suckled


e
f
g
Apo-Cp
B-Cp
Holo-Cp
Ponceau
132 kDa
130 kDa
95 kDa
% Band Intensity Apo-Cp
% Band Intensity Holo-Cp
B 5 50
ATP7A OE
Control
Resting
Lactating
Suckled
h
i
j
GPI-Cp
Na/K
135 kDa
100 kDa
% Band Intensity GPI-Cp

the corresponding treatment in the control cells. However the differences were only significant for the BCS/DPen treated cells ($p<0.05$) and not the 5 μM or 50 μM copper treated cells for each of the three models ($p>0.05$) (Fig 6.8c).

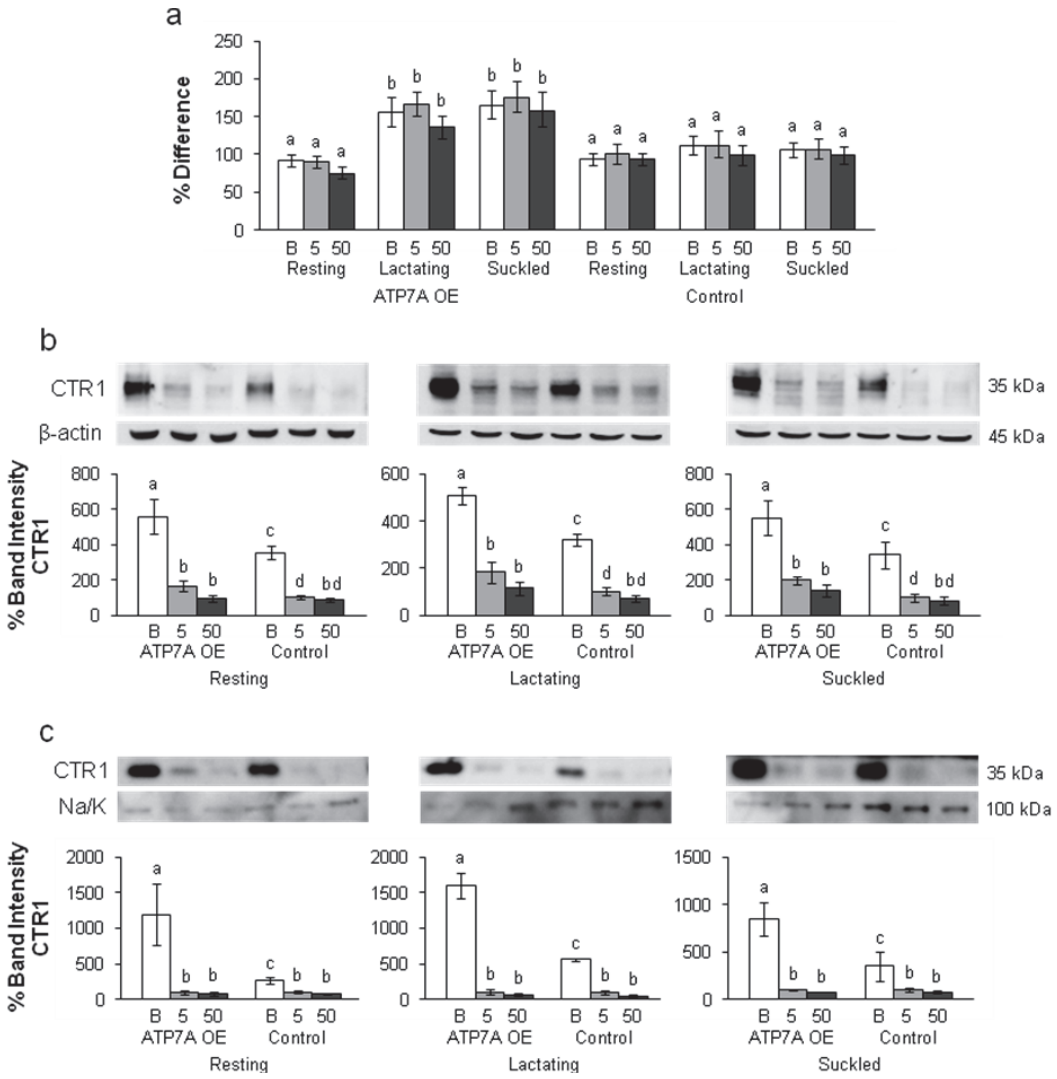
As observed in parental differentiated cells in chapter 3, CTR1 mRNA levels were unaltered by copper treatment. However, unlike the parental PMC42-LA cells, CTR1 mRNA was influenced by lactational hormones in the ATP7A overexpressing cells, increasing significantly in the lactating and suckled models ($p<0.05$). CTR1 protein levels decreased in response to elevated copper loads and surface biotinylation revealed a similar trend with more CTR1 protein being present at the plasma membrane when copper levels were depleted. Lactational hormones increased the levels of total and membrane bound CTR1 protein levels in ATP7A overexpressing cells, however CTR1 localisation determined by immunofluorescent confocal microscopy did not reveal any significant variation between treatment (data not shown). These results are consistent with those presented in chapter 3 for the parental PMC42-LA cells.

6.2.3.5 Intracellular copper levels increase in response to ATP7A overexpression in differentiated PMC42-LA cells

AAS analysis of copper levels revealed that ATP7A overexpression caused an increase in intracellular copper in all 50 μM copper treated cells as well as the 5 μM copper treated cells of the lactating and suckled models, compared to the control cells of corresponding treatment ($p<0.05$). The BCS/DPen treated and 5 μM copper treated resting model cells showed no difference in

Figure 6.8 CTR1 mRNA and protein in differentiated PMC42-LA cells

(a) Relative *mRNA* levels of CTR1 in response to ATP7A overexpression, copper and hormones obtained by qRT-PCR. The bar graph indicates the mean percentage differences ±SD compared to the 5 μM copper treated cells of the control resting model. *CTR1* mRNA levels were normalised against *β-actin*. B = BCS/DPen, 5 = 5 μM copper, 50 = 50 μM copper. (b) Representative Western blots of 60μg of protein extract from resting, lactating and suckled models comparing CTR1 bands in ATP7A overexpressing cells to control cells for each respective copper treatment. The β-actin blot demonstrates relative loading of samples. The CTR1 densitometry graph bars show mean intensity of ATP7A bands expressed as a percentage of arbitrary units ±SD compared to the 5 μM copper treatment of control cells, normalised against β-actin and represents data from three independent experiments. Statistical variation is indicated by different letters above graph bars ($p<0.05$). (c) Surface biotinylated CTR1 protein was analysed by Western blot. These representative Western blots of extracellularly biotinylated protein extract show the CTR1 band in ATP7A overexpressing and control cells of the resting breast model, lactating model and suckled model treated with BCS/DPen, 5 μM copper and 50 μM copper. Densitometric analysis showed mean intensity of CTR1 bands expressed as a percentage of arbitrary units ±SD compared to the 5 μM copper treatment of the control cells, normalised against Na/K ATPase and is representative of four independent experiments. Statistical variation is indicated by different letters above graph bars ($p<0.05$).


a
% Difference
250
200
150
100
50
0
B 5 50
Resting
Lactating
Suckled
ATP7A OE
Control
b
CTR1
β-actin
35 kDa
45 kDa
% Band Intensity CTR1
800
600
400
200
0
ATP7A OE
Control
Resting
Lactating
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c
CTR1
Na/K
35 kDa
100 kDa
% Band Intensity CTR1
2000
1500
1000
500
0

intracellular copper levels between the control and ATP7A overexpressing cells ($p>0.05$) (Fig 6.9). Analysis of XFM data revealed a similar trend (data not shown).

As demonstrated in differentiated parental PMC42-LA cells in chapter 3, the ATP7A overexpressing cells showed an accumulation of copper within the organoids compared to the surrounding monolayer of cells (Fig 6.10). No copper was detected in the BCS/DPen treated samples. The total fluorescence images indicate the position of cells and organoidal structures in each image.

6.2.4 ATOX1 expression is not influenced by copper, lactational hormones or elevated levels of ATP7A

QRT-PCR analysis revealed copper concentrations had no significant effect ($p>0.05$) on the expression of ATOX1 *mRNA* in the resting model, lactating model or suckled model of wild type PMC42-LA and ATP7A overexpressing PMC42-LA cells. The presence of hormones also had no significant effect on ATOX1 transcript ($p>0.05$) in wild type and ATP7A overexpressing cells. Also the overexpression of ATP7A had no impact on the mRNA levels of *ATOX1* ($p>0.05$) (Fig 6.11a).

A single band of approximately 7 kDa corresponding to the ATOX1 protein was detected across all samples treated with copper and lactational hormones in both the ATP7A overexpressing and control cells. Similar to the mRNA results, no differences in protein levels were detected in response to copper or lactational hormones. The overexpression of ATP7A also had no

Figure 6.9 Intracellular copper within differentiated PMC42-LA cells

AAS analysis of copper levels within control and ATP7A overexpressing cells of the resting, lactating and suckled models treated with BCS/DPen, 5 μM copper and 50 μM copper. B = BCS/DPen, 5 = 5 μM copper, 50 = 50 μM copper. Data is representative of three independent experiments. Statistical variation is indicated by different letters above graph bars ($p<0.05$).

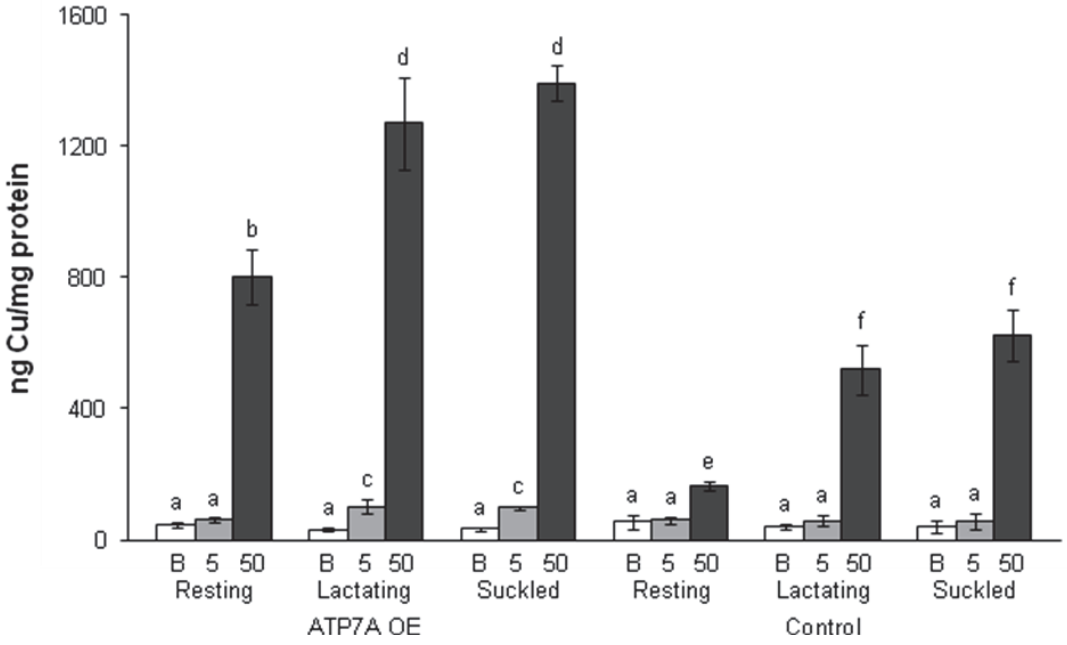
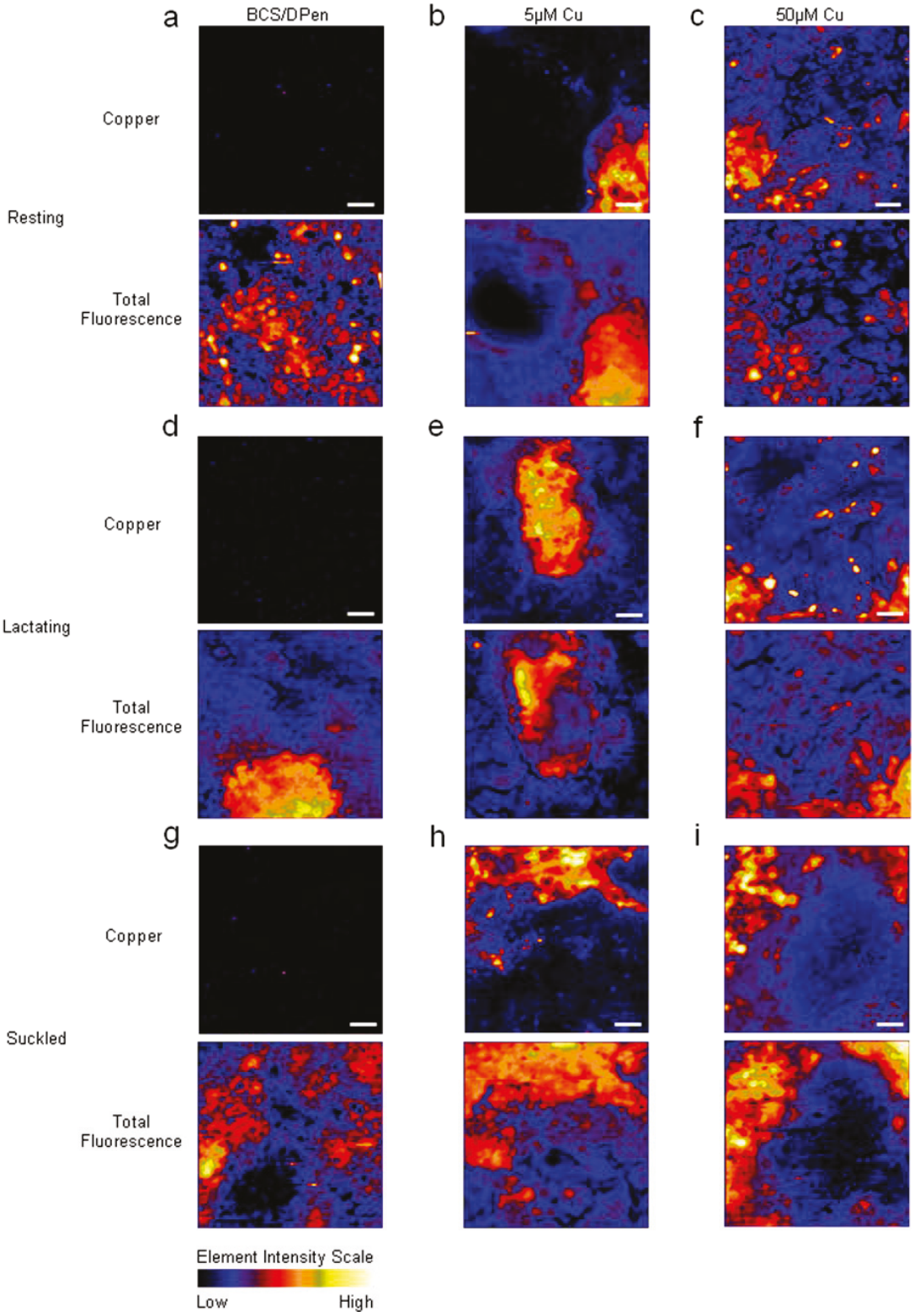

ng Cu/mg protein
1600
1200
800
400
0
a a b
a c d
a c d
a a e
a a f
a a f
B 5 50
Resting
Lactating
Suckled
Resting
Lactating
Suckled
ATP7A OE
Control

Figure 6.10 Copper localisation within the organoids of ATP7A overexpressing cells treated with copper and lactational hormones

XFM analysis revealed the distribution of copper within the organoids of copper and hormonally treated ATP7A overexpressing PMC42-LA cells. No copper was detected in the BCS/DPen treated cells of the resting model (a), lactating model (d) or suckled model (g), while copper was clearly visible in the 5 μM copper (b, e and h) and 50 μM copper (c, f and i) treated cells in each respective model. Total X-ray fluorescence images accompany the XFM copper images to show the position of each of the organoids. The colour scale bar shows the relative levels of copper from lowest (black) to highest (white). Length scale bar = 30 μm.


a
BCS/DPen
b
5μM Cu
c
50μM Cu
Copper
Resting
Total
Fluorescence
d
e
f
Copper
Lactating
Total
Fluorescence
g
h
i
Copper
Suckled
Total
Fluorescence
Element Intensity Scale
Low
High

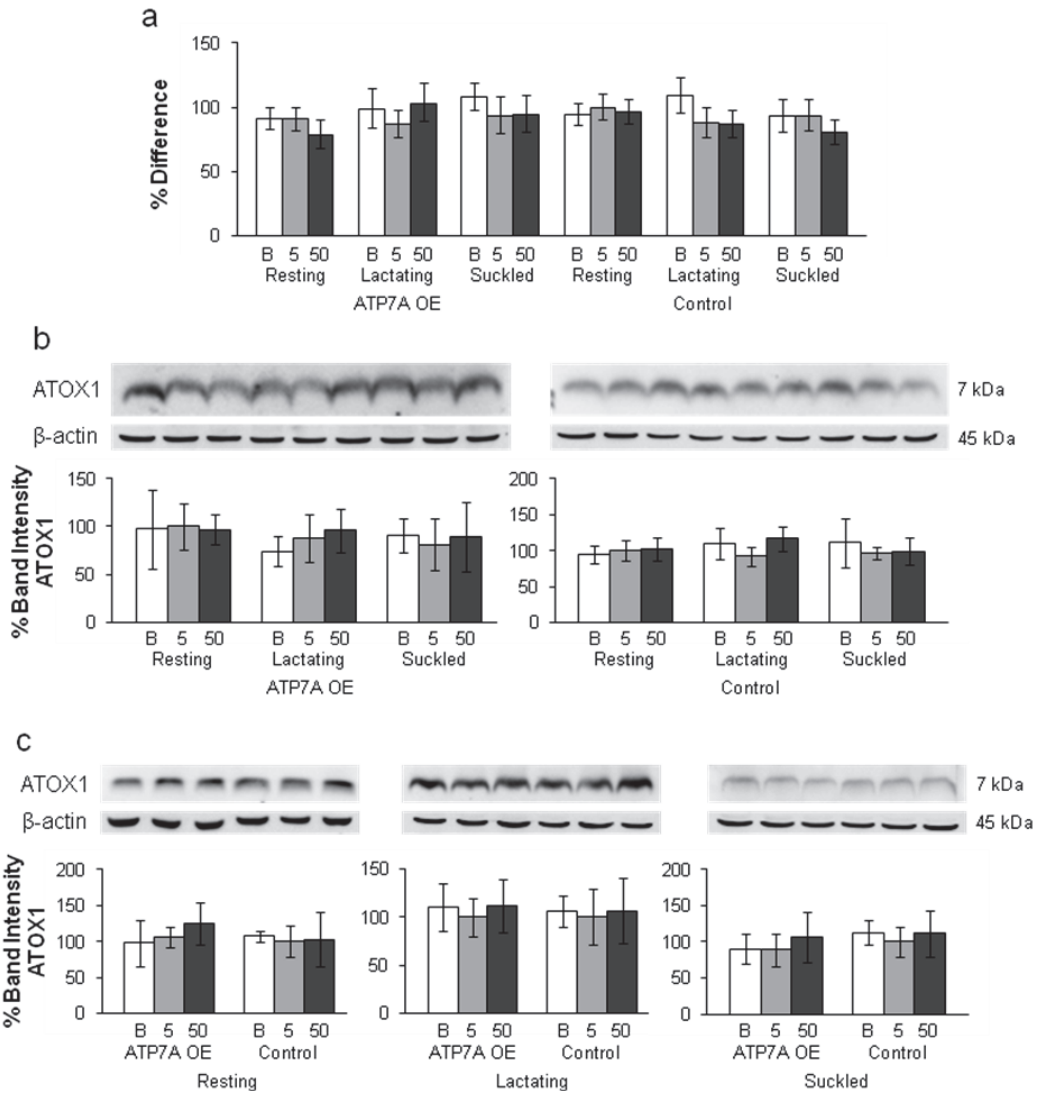
influence upon the levels of ATOX1 protein present in the cells ($p>0.05$) (Fig 6.11b and c).

6.2.5 ATP7A overexpression increased the levels of β2M protein, but not mRNA in PMC42-LA cells

Analysis of *β2M mRNA* by qRT-PCR revealed that copper, lactational hormones and ATP7A overexpression had no influence upon transcription of this gene ($p>0.05$) (Fig 6.12a). However the same cannot be said for the protein. While the addition of copper and lactational hormones did not influence β2M protein levels ($p>0.05$), overexpression of ATP7A caused a significant elevation in the level of β2M present within the cells ($p<0.05$). This effect was consistent in all three culture models and all three copper conditions (Fig 6.12b and c).

Figure 6.11 ATOX1 mRNA and protein expression levels in response to copper, lactational hormones and ATP7A overexpression

(a) Relative mRNA levels of *ATOX1* in response to copper, hormones and ATP7A overexpression obtained by qRT-PCR. The bar graph indicates the mean percentage differences ±SD compared to the 5 μM copper treated cells of the control resting model. *ATOX1* mRNA levels were normalised against *β-actin*. B = BCS/DPen, 5 = 5 μM copper, 50 = 50 μM copper. (b) Representative Western blots of 60μg of protein extract from ATP7A overexpressing and control cells showing ATOX1 bands in the resting breast model (lanes 1-3), lactating model (lanes 4-6) and suckled model (lanes 7-9) treated with BCS/DPen (lanes 1, 4 and 7), 5 μM copper (lanes 2, 5 and 8) and 50 μM copper (lanes 3, 6 and 9). (c) Representative Western blots of 60 μg of protein extract from resting, lactating and suckled models comparing ATOX1 bands in ATP7A overexpressing cells to control cells for each respective copper treatment. The β-actin blots demonstrate relative loading of samples. The ATOX1 densitometry graph bars show the mean intensity of ATOX1 bands expressed as a percentage of arbitrary units ±SD compared to the 5 μM copper treatment of the resting model (b) and the 5 μM copper treatment of the control cells (c), normalised against β-actin and is representative of three independent experiments.


a
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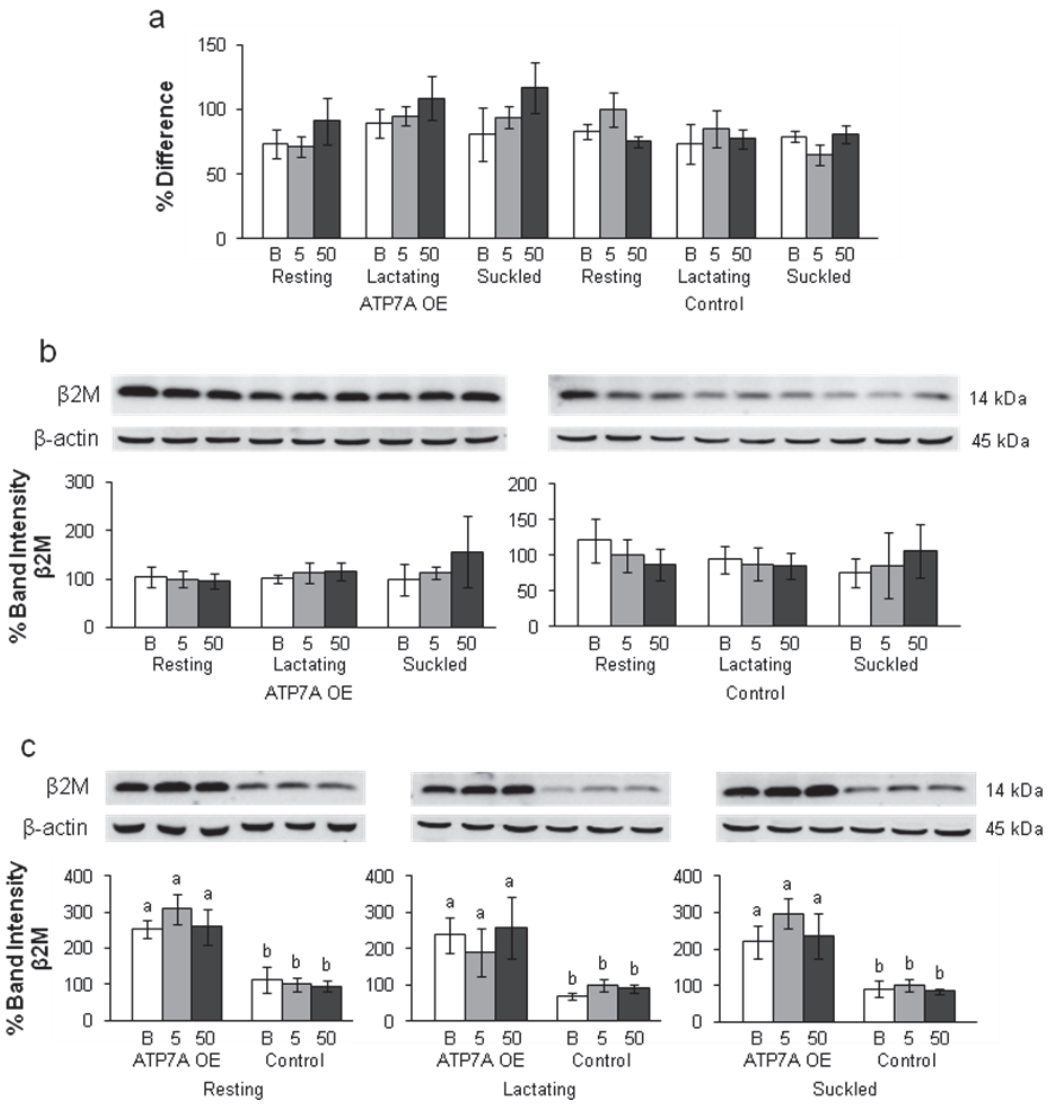
ATP7A OE
Control
b
ATOX1
β-actin
7 kDa
45 kDa
% Band Intensity ATOX1
200
150
c
Resting
Lactating
Suckled

Figure 6.12 β2M mRNA and protein expression in differentiated PMC42-LA cells

(a) Relative mRNA levels of *β2M* in response to copper, hormones and ATP7A overexpression obtained by qRT-PCR. The bar graph indicates the mean percentage differences ±SD compared to the 5 μM copper treated cells of the control resting model. *β2M* mRNA levels were normalised against *β-actin*. B = BCS/DPen, 5 = 5 μM copper, 50 = 50 μM copper. (b) Representative Western blots of 60 μg of protein extract from ATP7A overexpressing and control cells show β2M bands in the resting breast model (lanes 1-3), lactating model (lanes 4-6) and suckled model (lanes 7-9) treated with BCS/DPen (lanes 1, 4 and 7), 5 μM copper (lanes 2, 5 and 8) and 50 μM copper (lanes 3, 6 and 9). (c) Representative Western blots of 60μg of protein extract from resting, lactating and suckled models comparing β2M bands in ATP7A overexpressing cells to control cells for each respective copper treatment. The β-actin blot demonstrates relative loading of samples. β2M densitometry graph bars show mean intensity of β2M bands expressed as a percentage of arbitrary units ±SD compared to the 5 μM copper treatment of the resting model (b) and the 5 μM copper treatment of the control cells (c), normalised against β-actin and is representative of three independent experiments. Statistical variation is indicated by different letters above graph bars ($p<0.05$).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Resting
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ATP7A OE
Control
b
β2M
β-actin
14 kDa
45 kDa
% Band Intensity β2M
300
200
100
0
150
50
c
400
a
b

6.3 Discussion

The study described in chapter 5 examined the effects of ATP7A overexpression on copper levels and copper transporting proteins in undifferentiated PMC42-LA mammary cells. To further investigate copper homeostasis in human mammary epithelial cells, the differentiated models used in chapter 3 and 4 representing the resting, lactating and suckled mammary gland, were implemented to ascertain the responses of copper transporting proteins to copper, lactational hormones and ATP7A overexpression. The study was carried out to establish whether the changes observed in the undifferentiated cells, have similar implications in models that more closely represent physiological conditions of the resting and lactating mammary epithelium.

The results obtained from ATP7A overexpressing differentiated PMC42-LA cells, were similar to the data described in the previous three chapters showing responses to copper, lactational hormones and ATP7A overexpression. However there were still a few novel findings observed in the ATP7A overexpressing differentiated cultures that had not been detected in the undifferentiated cells.

In differentiated cells, ATP7A mRNA and protein expression levels were not responsive to extracellular copper treatments, consistent with the data of chapter 5. A slight increase in ATP7A expression was observed in response to lactational hormones, however this was not significant. Analysis of ATP7A localisation in the PMC42-LA cell models revealed some interesting results.

ATP7A responded to copper as it did in undifferentiated cells (Ackland et al., 1997, chapter 5), moving from a tight perinuclear localisation in depleted copper conditions and dispersing as copper concentrations increased. This phenomenon was seen in all differentiated PMC42-LA breast models; resting, lactating and suckled in both control and ATP7A overexpressing cells. However hormones also played a role in the trafficking of ATP7A as there was greater dispersion in the lactating model compared to that of the resting model, similar to the observations made of ATP7B (Michalczyk et al., 2008). This result correlates with differences observed in ATP7A localisation between resting human mammary tissue where the protein was found to be predominantly in the perinuclear region and lactating mammary tissue which showed a dispersed, cytoplasmic pattern (Ackland et al., 1999). These data suggest that the PMC42-LA resting and lactating models are more representative of the human breast than the undifferentiated PMC42-LA cells which showed no movement of ATP7A in response to lactational hormones (Ackland et al., 1997) and indicates that the full function of ATP7A trafficking only occurs in differentiated cells.

Some research has indicated that ATP7A localises apically as well as basolaterally in the mammary epithelial cells of hyperprolactinemic rats (Kelleher and Lonnerdal, 2006). This is not something that was observed in the PMC42-LA cells. The localisation of ATP7A in these cells, while dispersed, was more prominent in the basolateral part of the cell. This agrees with research which showed that ATP7A is targeted towards the basolateral membrane via its dileucine motif (Greenough et al., 2004). The basolateral localisation observed in PMC42-LA cells is also more reminiscent of research

conducted on ATP7A overexpressing mice which showed predominantly basolateral localisation (Llanos et al., 2008).

Surface biotinylation analysis of ATP7A supported the immunolocalisation results. A greater amount of ATP7A was observed at the plasma membrane in response to copper, while very little was detected in the chelated samples. The addition of hormones also increased the presence of ATP7A at the plasma membrane.

The results obtained are consistent with the concept that ATP7A is mobilised to the basolateral membrane during lactation and functions to transport copper back into the maternal bloodstream, protecting a suckling infant from receiving high copper levels in milk. The hormonally induced dispersion of ATP7A seems counterproductive in relation to the increased presence of CTR1 at the plasma membrane in response to hormones observed in chapter 3. However the cells may be increasing copper uptake by CTR1 at the basal membrane to enhance the movement of copper into the cell and then into the milk via ATP7B and ceruloplasmin (Cp), while ATP7A is effluxing copper across the basolateral membrane when intracellular copper levels become too high or when copper secretion across the apical membrane needs to be impeded to prevent the build up of high milk copper levels.

Responses of CTR1 in differentiated ATP7A overexpressing cells were analysed as described in chapter 3 for PMC42-LA cells expressing only endogenous ATP7A and comparisons were made between the two cell

types. The addition of copper, hormones or presence of excess ATP7A had no observable effect upon the localisation of CTR1 when analysed by immunofluorescence microscopy (data not shown); however surface biotinylation did indicate that there was less protein at the plasma membrane when cells were exposed to elevated copper. This was also reflected in the total CTR1 protein levels of the cells. Copper depletion increased CTR1 protein levels within the cells and at the plasma membrane, while copper caused levels to reduce. Like copper chelation, the addition of lactational hormones and the overexpression of ATP7A increased the levels of CTR1. These data are consistent with the proposed model in which CTR1 enables an influx of copper from the basolateral membrane of the cells. The increase in CTR1 expression in cells exposed to copper chelation may be induced to elevate copper uptake in depleted conditions, while in copper loaded cells, CTR1 is down-regulated to prevent the uptake of toxic levels of copper. The elevation of CTR1 in response to ATP7A overexpression may occur to balance increased copper efflux caused by the additional ATP7A protein. However the lactational hormone response of CTR1 may be initiated to increase copper uptake into the cell which would allow for the movement of additional copper across apical side of the cells into the milk. During lactation and suckling, additional copper is required in the lumen of the alveolus to supply a suckling infant with adequate copper. For this to occur, the copper must first be taken up into the cells from the blood, a role performed by CTR1.

Most of the CTR1 responses to copper, lactational hormones and the overexpression of ATP7A were similar to those presented previously in

chapter 3 and 5. However, the overexpression of ATP7A in combination with lactational hormones increased the levels of *CTR1* mRNA. This effect was not observed previously as individually lactational hormones and ATP7A overexpression had no effect on *CTR1* mRNA. It is not clear whether the increase in mRNA translates into protein changes. While CTR1 protein levels were increased in hormonally treated ATP7A overexpressing cells, there was also a higher level of CTR1 protein in hormonally treated control cells and in non-hormonally treated ATP7A overexpressing cells. This indicates that protein levels were influenced by hormones and ATP7A overexpression independently, whereas the mRNA required the presence of both to be elevated. The increase in CTR1 protein in response to hormones may be a consequence of reduced degradation of CTR1 protein rather than increased transcription, as this increase was not accompanied by transcriptional differences in the control cells. If the increase in *CTR1* transcription in ATP7A overexpressing cells does translate into protein changes, it is possible that the posttranslational degradation has a greater influence and prevents the protein levels increasing significantly. Additionally, densitometric analysis of western blot bands is rather cruder than the analysis of mRNA by qRT-PCR which is more sensitive and subtle changes can be detected more easily.

In this study it was observed that *ATP7B* mRNA was not affected by lactational hormones in PMC42-LA cells. Upon overexpression of ATP7A, this trend was altered as *ATP7B* mRNA increased in the presence of ATP7A overexpression and lactational hormones. This change is not solely a response to ATP7A overexpression as the ATP7A overexpressing resting model which is not exposed to lactational hormones showed no difference in

ATP7B expression compared to the control cells. Likewise it is not solely an effect of the lactational hormones as ATP7B expression did not respond to hormonal treatment in the parental PMC42-LA cells. While there was a clear increase in *ATP7B* mRNA levels in response to ATP7A overexpression in conjunction with hormones, it was not possible to determine whether this correlated to an increase in protein levels. The ATP7B protein analysis results were inconsistent and so a final conclusion as to whether the protein levels reflected mRNA levels could not be drawn.

The increases in CTR1 and ATP7B mRNA in response to ATP7A overexpression and lactational hormones may be occurring to oppose the effect of ATP7A. It is possible that there are non-copper induced signalling mechanisms that are initiated by ATP7A overexpression that cause *CTR1* and *ATP7B* mRNA expression to increase, resulting in enhanced uptake of copper across the basolateral membrane into the cell via CTR1, which then allows the increased levels of ATP7B to facilitate greater copper efflux across the apical membrane for secretion into the milk, thus reducing the effect of ATP7A overexpression.

In addition to direct regulation by hormones and ATP7A overexpression, copper transporters can also be influenced by varying copper levels. The real time PCR data indicated that *ATP7B* is responsive to copper levels. In spite of the *ATP7B* promoter region containing four metal response elements (Cullen et al., 2003), most previous research on human tissue, rats, mice and human cultured cells, has shown no change in response to copper (Gambling et al., 2004, Llanos et al., 2008, Michalczyk et al., 2008, Michalczyk et al.,

2000, Prohaska, 2008, Schilsky et al., 1998). However there have been two studies which did show some different responses. The first showed increase in mRNA but not protein in the livers of pups fed copper supplemented diets (Bauerly et al., 2005). The second study showed that during the early phase of lactation when breast plasma copper levels were highest, mammary gland *ATP7B* mRNA was at its lowest. As copper levels reduced with the progression of lactation, *ATP7B* levels increased (Kelleher and Lonnerdal, 2006). The second study correlates with the data presented here and is most relevant as it also pertains to the mammary gland. The protein levels of the second study did not correlate with the mRNA as they identified a slight, but statistically significant down-regulation of ATP7B protein in the latter stages of lactation, opposite to the mRNA results (Kelleher and Lonnerdal, 2006). The research presented in chapter 5 on PMC42-LA cells grown in flasks showed no change in *ATP7B* mRNA in response to copper in either wild type or ATP7A overexpressing cells, however one key difference is that the data presented in this chapter was performed on differentiated cultures. It is possible that *ATP7B's* transcriptional regulation is more sensitive to copper in differentiated cells. Increasing copper levels caused significant reduction in *ATP7B* mRNA in all ATP7A overexpressing models, as well as the lactating model of the control cells. The resting and suckled control models did not show statistically significant down-regulation, however there was a trend showing a decrease as copper levels rose. This result appears contrary to ATP7B's role as a copper efflux pump, in which an increase in ATP7B would be expected with an increase in elevated copper to assist with the efflux of high levels of copper. However, due to ATP7B's apical localisation, an increase would result in the transport of more copper into the milk. This

would maintain copper levels in the mammary epithelial cells, but would elevate milk copper levels which may have adverse effects upon the suckling infant. Reducing the level of ATP7B when copper is elevated would reduce this risk, however the cells would need to rely solely on ATP7A to remove excess copper from the cells across the basolateral side of the cell. Given that most cells types in the human body only use ATP7A as a copper efflux pump (Vulpe et al., 1993), this should be sufficient for the mammary gland as well. The opposite response was found in copper chelated samples which showed elevated *ATP7B* mRNA levels. While not significant, levels were consistently higher than in the 5 μM copper control cells. This would suggest that copper deficient cells are increasing ATP7B to maintain copper secretion into milk; preventing the suckling infant from receiving inadequate amounts of copper.

Unfortunately the ATP7B mRNA changes observed in response to copper could not be conclusively confirmed by protein analysis. Some data showed distinct changes in ATP7B levels in response to copper, with BCS/DPen levels being highest, while the copper treated samples expressed much less, almost undetectable levels of protein; this is similar in trend to the mRNA. These blots showed a reduction in ATP7B protein in response to copper in all cell models (resting, lactating and suckled) in both cell types (ATP7A overexpressing and controls). In contrast to this, there were some blots which showed fewer differences between copper treatments, or sometimes variation which could be considered random, showing no distinct trends at all. The densitometric analysis of these inconsistent results did not reveal any trend or significant changes that could be correlated with the mRNA data.

Two ATP7B antibodies have been used for this work, one custom made, one commercially available, and both yielded poor results. The custom made antibody produced multiple bands on the blot and so it was difficult to determine which band was ATP7B. The band that was closest to the predicted ATP7B size was not consistent in intensity and almost undetectable in some of the samples. The commercially available ATP7B antibody worked effectively for the undifferentiated cells, giving clear results which were presented in chapter 5; however this was not the case for differentiated cells. Detection of the protein by Western blot was complicated by the difficulties of sample preparation for the differentiated cells. These cells were grown on a layer of EHS matrix. When the cells were collected, some of this matrix contaminated the samples, adding to the total protein estimations. This also made it more difficult to lyse and homogenise the cells in a small volume of lysis buffer as it increases the viscosity of the sample. Once the sample was homogenous, the concentration was lower than desired, thus it was difficult to load more than 60 µg of protein, where it may be more useful to load 100-120 µg for easier detection of this protein. To further analyse ATP7B in these cells, another antibody that is more sensitive would be required for analysis of ATP7B protein in differentiated PMC42-LA cells.

The ATP7B mRNA demonstrated some unexpected results, however the localisation of the protein was consistent with those observed previously (Michalczyk et al., 2008, chapter 5). ATP7B resided near the TGN in copper depleted samples and dispersed with the addition of copper and lactational hormones (data not shown). This is consistent with its role as an efflux pump,

receiving copper in the TGN from ATOX1 and trafficking towards the plasma membrane where it enables the efflux of copper into milk.

ATOX1, the copper chaperone that delivers copper to, ATP7A and ATP7B, showed no variation in response to copper levels or ATP7A overexpression in undifferentiated PMC42-LA, as demonstrated in chapter 5. The results from the differentiated cultures were no different, with the copper and hormonal treatments and also ATP7A overexpression having no influence upon the mRNA or protein levels in PMC42-LA cells. There has been little research analysing the effect of copper on ATOX1 and none on the effect of lactational hormones. However, it has been shown that the altered copper status of mice fed iron deficient and also copper deficient diets has little impact on ATOX1 expression in the placenta and liver (Andersen et al., 2007, Gambling et al., 2004). As stated previously (Lutsenko et al., 2007, chapter 5), there is an excess of *ATOX1* transcript with respect to *ATP7A* and *ATP7B* and so perhaps alteration of ATOX1 expression is unnecessary as levels are always sufficient when the demand for loading copper onto the ATPases is high.

The experiments described in chapter 4 and 5 have clearly demonstrated that the secreted form of Cp and the membrane bound glycosylphosphatidylinositol-linked Cp (GPI-Cp) are influenced by copper and lactational hormones in undifferentiated and differentiated cells and by ATP7A overexpression in undifferentiated cells. The results obtained from differentiated ATP7A overexpressing cultures were consistent with the previous data. Increases in Cp transcription and secretion in response to

lactational hormones ensures an adequate supply of Cp into the milk when demand is high. Additionally, the presence of copper is important as Cp is inactive in its apo form (when it does not have copper bound) and unable to oxidise iron, therefore copper deficiency would impact upon not only copper, but also the supply of iron to a suckling infant as well. A clear understanding of the mechanisms causing Cp up-regulation in response to ATP7A overexpression has yet to be determined, as discussed in chapter 5. However it is possible that similar to the increases in CTR1 and ATP7B, the increase in Cp is intended to counteract the effect of additional ATP7A moving copper across the basolateral membrane, promoting sufficient copper flux in the direction of the milk. The role of GPI-Cp in lactation is unknown. It is possible that GPI-Cp aids the efflux of iron, or perhaps the oxidation of iron which would enable iron binding to lactoferrin; similar to the role performed by sCp (de Domenico et al., 2007, Jeong and David, 2003, Kono et al., 2006a, Sabatucci et al., 2007, Sokolov et al., 2007, Zakharova et al., 2000). Cp localisation in differentiated ATP7A overexpressing cells remained unchanged compared to the control cells and was not influenced by copper or lactational hormones (data not shown).

The research described in chapter 5 demonstrated that an increase in expression of ATP7A resulted in elevated levels of β2-microglobulin (β2M). This is likely the result of the ATP7A effluxing more copper ions which would normally be available to destabilise the β2M protein (Deng et al., 2006). β2M is known to play a role in iron uptake, where it binds to HFE which enhances transferrin receptor's ability to bind and take up iron loaded transferrin (Bhatt et al., 2009, Bhatt et al., 2010, Enns, 2001, Vecchi et al., 2010). However, a

number of studies have also outlined the impact of copper in destabilisation of β2M which causes the formation of the amyloid plaques that aggregate in joints and connective tissue causing Dialysis-Related Amyloidosis (Calabrese and Miranker, 2009, de Lorenzi et al., 2008, Deng et al., 2006). Additionally, some research has implicated β2M in the growth and proliferation of cells (Huang et al., 2008, Huang et al., 2006, Josson et al., 2011, Rowley et al., 1995, Yang et al., 2006) and that this may be the underlying cause of some of the unexpected changes.

Copper was observed to accumulate in differentiated ATP7A overexpressing cells compared to controls. Strangely, this response contradicts the previous data from undifferentiated ATP7A overexpressing PMC42-LA cells described in chapter 5 and is not consistent with ATP7A's role as a copper efflux protein, in which overexpression should decrease copper levels in the cell. It was noted as the ATP7A overexpressing cells grew in culture, that the colour of the medium was different to the controls at the conclusion of the experiment, even though the cells for both cultures were seeded at the same density and in the same volume of growth medium. The phenol red pH indicator in the growth medium was consistently more yellow in the ATP7A overexpressing cells compared to the controls, suggesting that these cells had lowered the pH of the medium as a result of creating more metabolic waste products, which is likely a consequence of increased growth and proliferation. This was observed in all three models treated with BCS/DPen, 5 μM or 50 μM copper. While the culture medium of ATP7A overexpressing cells was consistently more yellow than the control medium, there was no noticeable difference in colour between the copper or hormonal treatments.

The growth medium colour correlated with the β2M protein levels which increased in ATP7A overexpressing cells. It has been shown previously that copper and also β2M can promote cellular proliferation (Huang et al., 2008, Huang et al., 2006, Itoh et al., 2008, Josson et al., 2011, Rowley et al., 1995, Yang et al., 2006). It is possible that β2M influences pathways that enable increased copper uptake to enhance cellular growth. For example, β2M activates the MAPK/ERK and PI3K/Akt pathways (Huang et al., 2010, Yang et al., 2006), and influences the levels of cyclin A and cyclin D1 (Huang et al., 2006); all of these factors are involved in the growth and proliferation of cells. MAPK/ERK and PI3K/Akt have also been shown to increase with the addition of copper (Barthel et al., 2007, Samet et al., 1998, Veldhuis et al., 2009, Walter et al., 2006). However, BCS/DPen treated cells have very little available copper that would make copper enhanced proliferation in these cells very unlikely.

An alternate proposal to the theory that β2M induces growth and proliferation by altering the copper status of the cell is that β2M caused an increase in growth and proliferation via non-copper mediated pathways, thus explaining the consistency of the colour of the growth medium in all copper treatments. It was shown that blocking β2M with a β2M antibody, activated the MAP kinase JNK pathway which initiates stress responses, growth arrest and apoptosis, while suppressing the MAPK/ERK and PI3K/Akt pathways (Huang et al., 2010, Yang et al., 2006). This indicates that under normal circumstances, β2M activates the MAPK/ERK and PI3K/Akt pathways, promoting growth and prolifteration, while at the same time suppressing growth repression and apoptotic MAPK/JNK pathways. Hence, increased

expression of β2M in ATP7A overexpressing cells, should lead to an increased rate of cellular growth and proliferation.

Differing growth rates between ATP7A overexpressing and control cells alters the dynamics of the culturing system which has implications for maintaining experimental consistency. It was identified during method development of the differentiated cultures, that the degree of organoid formation is influenced by the seeding density of the cells. It was also noted that the ATP7A overexpressing cultures formed more and also larger organoids than the control cultures when seeded at the same density. This is likely a result of differing cellular growth and proliferation rates, which change the culture density as the experiment progressed. It was also noted that the monolayer cells have centrally rather than basally localised nuclei (Ackland et al., 2001) and so it is possible that they are not as polarised or differentiated as the organoidal cells. If true, the organoidal cells may behave more like luminal epithelial cells than the monolayer cells and may take up more copper with the intention of secreting it across the apical membrane into the lumen of the organoid. As a result, the organoidal cells may have slightly higher intracellular copper levels than the monolayer cells. If this occurs, the ATP7A overexpressing cells, which have more and also larger organoids than the control cultures, would have a higher proportion of organoidal cells than the controls. This could lead to the ATP7A overexpressing cultures having more cells with a higher concentration of intracellular copper in them; masking an observable copper reduction caused by the increased expression of ATP7A. The XFM data also showed higher copper levels within the organoidal structures. In chapter 3 it was concluded that most of this copper

is within the lumen of the organoids. The elevation in luminal copper could also be caused by the ATP7A overexpressing cultures having larger organoids, which would have more cells that are able to secrete copper into the lumen. This would result in the ATP7A overexpressing cultures having more copper in the lumen of their organoids than the control cultures.

6.4 Conclusions

The overexpression of ATP7A in a differentiated cell culture model has facilitated the study of copper homeostatic disturbances in a system more reminiscent of the physiological conditions encountered in the mammary gland. ATP7A is proposed to have a protective role in the lactating mammary gland, moving copper across the basolateral membrane back to the maternal bloodstream. The addition of lactational hormones caused ATP7A to disperse to the membrane. In conjunction with the overexpression of ATP7A, lactational hormones also induced expression of proteins associated with the movement of copper towards the apical surface of the mammary epithelial cells. It was also shown for the first time that *ATP7B* levels were altered by exposure to increased copper levels, a response thought to help tightly regulate copper delivery to milk. These changes which were not observed in undifferentiated cultures, suggest that differentiated cells may regulate copper more stringently, particularly in the presence of lactational hormones. This attribute may be essential to maintain appropriate copper levels in the secreted milk, enabling sufficient nourishment of a suckling infant, while at the same time preventing exposure to toxic copper levels that may be harmful.

CHAPTER 7

Conclusion

Maintaining strict copper homeostasis is crucial for proper functioning of all types of cells and tissues in the body. Extensive research has been conducted to help explain the role of individual copper transporters in a variety of cells. However less is known about the copper fluxes in complex systems containing more than one copper efflux protein. It is important to gain an understanding of these more complex systems as they are present in organs essential to the nourishment of developing foetuses and neonates; the placenta and mammary gland. The mammary gland produces milk which is an important dietary source for neonates. Adequate copper secretion into milk is essential for neonatal growth and development.

Copper homeostasis in mammary epithelial cells is controlled by a small number of influx and efflux proteins. CTR1 controls copper uptake from the maternal blood stream (Kelleher and Lonnerdal, 2003, Kelleher and Lonnerdal, 2006, Llanos et al., 2008). Once inside the cells, copper is delivered to ATP7A or ATP7B by copper chaperone ATOX1 (Banci et al., 2007, Banci et al., 2009, Strausak et al., 2003, Tanchou et al., 2004, van Dongen et al., 2004). ATP7A and ATP7B are key components of cellular copper efflux. ATP7A is important in removing excess copper across the basolateral membrane back into the maternal bloodstream, while ATP7B is important in the deliverly of copper to Cp for secretion across the apical membrane into milk (Kelleher and Lonnerdal, 2006, Llanos et al., 2008, Platonova et al., 2007, Tsymbalenko et al., 2009). MT also plays an important role in preventing copper toxicity by binding and storing excess cellular copper (Mehta et al., 2006, Sutherland and Stillman, 2011, Urani et al., 2003). The current research has extensively studied the responses of these

proteins to various copper levels, to the increases in hormone levels experienced during lactational and neonatal suckling and finally in response to the overexpression of copper efflux protein ATP7A.

This research has unveiled some interesting findings. Initially, the differentiated culture models used previously that represent the resting and lactating mammary gland, were further developed to create an environment that simulated the suckling of a feeding infant. Analysis of copper levels in these culture models revealed that lactational hormones increased copper uptake. This was most clearly observed in cells treated with high copper representing the levels present in blood during lactation. This result indicates that upon hormonal stimulation, the cells are absorbing more copper, possibly to enhance the secretion of copper into milk. It was also observed that the organoidal structures that are formed in culture were behaving like the lobules of a mammary gland; secreting copper into the lumen of the organoids. This demonstrates that the model is functional, secreting trace metals and milk proteins across the apical membrane of the polarised cells into the luminal cavity of the three dimensional organoid structures.

Upon analysis of the individual transporters, CTR1 expression and localisation was found to be influenced by copper and lactational hormones. Total CTR1 protein levels and its presence at the membrane were reduced with the addition of copper which is intended to decrease copper uptake. As an uptake molecule, it is important that CTR1 can regulate copper fluxes in response to its surroundings. It is crucial for cellular survival to prevent excess uptake of copper which can lead to the production of free radicals.

However, when intracellular copper levels were elevated, the cells increased the expression of metallothionein 1 and 2, which can bind excessive copper and protect the cells from copper toxicity. The addition of lactational hormones increased total and membrane bound CTR1 levels. This increase is likely intended to enhance the movement of copper into milk secretions.

Regulating copper efflux is as important as regulating copper uptake. In the mammary epithelial cells copper can be effluxed across the basolateral membrane by ATP7A or across the apical membrane by ceruloplasmin (Cp). Ceruloplasmin (Cp) is the main carrier of copper in milk and control of its release from mammary epithelial cells is an important factor in regulating milk copper levels. Copper levels had little influence upon Cp expression and secretion, however its presence determined whether the Cp protein was copper loaded and ferroxidase active. Lactational hormones initiated an increase in Cp transcription and secretion. This could be a response to increased demand, as lactation and suckling would require a high rate of Cp secretion to maintain adequate copper supply to the milk.

ATP7A binds copper in the TGN and traffics to the plasma membrane where it is able to efflux copper from the cell. Its dispersion away from the TGN and presence at the plasma membrane is regulated by copper. As copper levels rise, more ATP7A moves towards the membrane, helping to maintain appropriate cellular copper concentrations. The addition of lactational hormones also increased the dispersion of ATP7A and its presence at the plasma membrane. In mammary epithelial cells, ATP7A is most likely involved in copper efflux across the basolateral membrane. Increasing

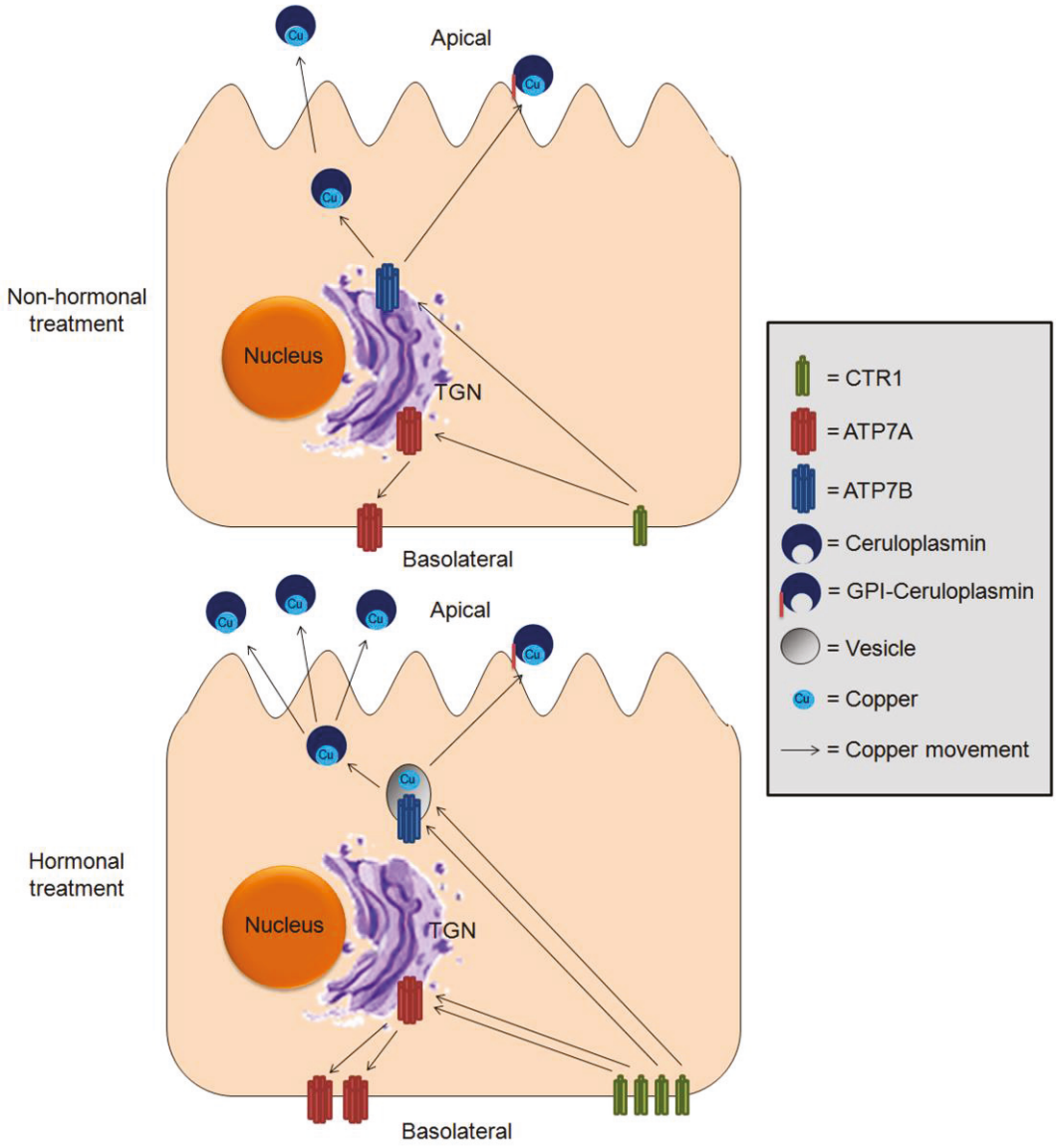
copper efflux across the basolateral membrane in response to lactational hormones is counterproductive to the increased copper flux into the milk caused by CTR1 and Cp. It is possible that the response to lactational hormones is designed to increase the total flux of copper in and out of the cell. This model would suggest that copper is always moving back and forth across the basolateral membrane and when required it can be redirected towards the apical membrane for secretion into milk.

ATP7B may assist the movement of copper towards the milk. A decline in ATP7B mRNA was observed in response to copper which may also be the case for protein levels, indicating that ATP7B may be important in regulating copper transport across the apical membrane. When copper levels are depleted ATP7B expression is increased in an attempt to raise the milk copper content, making it more nutritious. Upon copper overload, ATP7B is down-regulated to protect the suckling infant from being exposed to high and potentially toxic copper levels. ATP7B most likely regulates milk copper in conjunction with Cp. ATP7B was not shown to be present at the plasma membrane of cells, however it was shown to directly interact with Cp. Figure 7.1 demonstrates graphically the changes induced by lactational hormones.

These data were further supported by the experiments using ATP7A overexpressing cells which caused an up-regulation of *Cp*, *CTR1* and *ATP7B* mRNA in differentiated cells in the presence of lactational hormones. These responses were not copper related as these genes did not respond to altered copper levels. Results suggest that there are non-copper related pathways which are activated by the presence of additional ATP7A. The hypothesised

Figure 7.1 Lactational hormone induced changes of copper transporting proteins

This schematic diagram of copper transporting proteins in mammary epithelial cells compares non-hormonal to hormonal conditions in the presence of 5 μM copper. The diagram shows the localisation of proteins with regard to the apical and basolateral membranes of mammary epithelial cells. Arrows indicate the movement of copper through the cells.


Apical
Non-hormonal treatment
Nucleus
TGN
Basolateral
Apical
Hormonal treatment
Nucleus
TGN
Basolateral
= CTR1
= ATP7A
= ATP7B
= Ceruloplasmin
= GPI-Ceruloplasmin
= Vesicle
= Copper
= Copper movement

pathways counteract the effect of ATP7A by up-regulating the proteins involved in moving copper in the direction of apical membrane. This is particularly evident when lactational hormones are present as there is increased requirement for copper to be moved into the milk.

Glycosylphosphatidylinositol-linked Cp (GPI-Cp) was detected for the first time in mammary epithelial cells. It was detected at the plasma membrane and proved unresponsive to copper and lactational hormones, although its expression was elevated in response to ATP7A overexpression. The GPI linked form of Cp is membrane bound is likely to have a role in oxidation and efflux of iron (Jeong and David, 2003, Kono et al., 2006a). sCp has ferroxidase activity, delivers iron to transferrin and interacts with lactoferrin. GPI-Cp has similar ferroxidase activity and it is possible the both GPI-Cp and sCp play an important role in loading lactoferrin with iron.

In future it will be important to verify the ATP7B protein results and determine its responsiveness to copper which will help to obtain a more complete understanding of the copper transport pathways in mammary epithelial cells under physiological conditions. Further analysis of sCp and particularly GPI-Cp, which has received little attention in the literature, will further elucidate their roles and their impact upon the copper and iron composition of milk. In particular, it will be beneficial to obtain antibodies specific to apo and holo-Cp and also GPI-Cp to identify any potential differences in localisation. It would also be worthwhile attempting to isolate GPI-Cp in its non-reduced form enabling analysis of the apo and holo forms of this protein. Further mRNA analysis by qRT-PCR using specific primers to sCp and GPI-Cp would

divulge more information on the relative levels of the two forms of Cp. This may help elucidate the importance of GPI-Cp in mammary epithelial cells, to determine whether it plays key roles in copper and iron homeostasis or merely a supporting role to sCp.

Further analysis identifying the pathways influenced by ATP7A that initiate up-regulation of CTR1, ATP7B and Cp should also be performed. It would be interesting to see whether these pathways affect other aspects of cellular function outside of the copper transport system. In addition it would also be interesting to silence ATP7A to determine whether it imposes an inhibitory action upon these three genes.

If it is confirmed that ATP7A initiates transcriptional pathways by some mechanism, it would be interesting to see if this effect could be replicated in other mammary and non-mammary gland cells lines, to determine whether these effects are mammary epithelial cell-specific or universal among all cells. If these effects occur in other cell lines it would be informative to increase the expression of other copper transporting genes, in particular ATP7B which has a similar sequence and structure to ATP7A, and determine whether overexpression of these genes also influence transcription.

The findings from this research have underlined the importance of copper transporting proteins in maintaining cellular copper homoeostasis and provided insights into their role in regulating copper delivery into milk. It was shown that during lactation there is an emphasis on enhancing the movement of copper into the milk by increasing uptake by CTR1 and efflux

by Cp, while ATP7A and MT may have protective roles associated with preventing the secretion of toxic copper levels into the milk.

References

ACKLAND, M. L., ANIKIJENKO, P., MICHALCZYK, A. & MERCER, J. F. B. (1999) Expression of Menkes Copper-Transporting ATPase, MNK, in the Lactating Human Breast: Possible Role in Copper Transport into Milk. *J. Histochem. Cytochem.,* 47, 1553-1561.

ACKLAND, M. L., CORNISH, E. J., PAYTNER, J. A., GRIMES, A., MICHALCZYK, A. & MERCER, J. F. B. (1997) Expression of Menkes Disease Gene in Mammary Carcinoma Cells. *Biochem. J.,* 328, 237-243.

ACKLAND, M. L., MICHALCZYK, A. & WHITEHEAD, R. H. (2001) PMC42, A Novel Model for the Differentiated Human Breast. *Exp. Cell Res.,* 263, 14-22.

ACKLAND, M. L., WARD, J., ACKLAND, C. M., GREAVES, M. & WALKER, M. (2003) Extracellular Matrix Induces Formation of Organoids and Changes in Cell Surface Morphology in Cultured Human Breast Carcinoma Cells PMC42-LA. *In Vitro Cell. Dev. Biol. Anim.,* 39, 428-433.

AFTON, S. E., CARUSO, J. A., BRITIGAN, B. E. & QIN, Z. (2009) Copper Egress is Induced by PMA in Human THP-1 Monocytic Cell Line. *Biometals,* 22, 531-539.

AGGETT, P. J. (1985) Physiology and Metabolism of Essential Trace Elements: An Outline. *Clin. Endocrinol. Metab.,* 14, 513-543.

ALA, A., WALKER, A. P., ASHKAN, K., DOOLEY, J. S. & SCHILSKY, M. L. (2007) Wilson's disease. *Lancet,* 369.

ALBERA, E. & KANKOFER, M. (2009) Antioxidants in Colostrum and Milk of Sows and Cows. *Reprod. Domest Anim.,* 44, 606-611.

ALLEN, T. M., MANOLI, A. & LAMONT, R. L. (1982) Skeletal Changes Associated with Copper Deficiency. *Clin. Orthop. Relat. Res.,* 168, 206-210.

AMARAVADI, R., GLERUM, D.M. & TZAGOLOFF, A. (1997) Isolation of a cDNA Encoding the Human Homolog of COX17, a Yeast Gene Essential for Mitochondrial Copper Recruitment. Hum. Genet., 99, 329–333.

ANDERSEN, H. S., GAMBLING, L., HOLTROP, G. & MCARDLE, H. J. (2007) Effect of Dietary Copper Deficiency on Iron Metabolism in the Pregnant Rat. *Br. J. Nutr.,* 97, 239-246.

ANI, M. & MOSHTAGHIE, A. A. (1990) Comparative Effects of Estradiol, Progesterone and Testosterone on the Level of Rat Serum Ceruloplasmin. *Journal of Islamic Academy of Sciences,* 3, 143-145.

ARAYA, M., MCGOLDRICK, M. C., KLEVAY, L. M., STRAIN, J. J., ROBSON, P., NIELSEN, F., OLIVARES, M., PIZARRO, F., JOHNSON, L. & POIRIERX, K. A. (2001) Determination of an Acute No-Observed-Adverse-Effect Level (NOAEL) for Copper in Water. *Regul. Toxicol. Pharmacol.,* 38, 389-399.

ARMENDARIZ, A. D., GONZALEZ, M., LOGUINOV, A. V. & VULPE, C. D. (2004) Gene Expression Profiling in Chronic Copper Overload Reveals Upregulation of Prnp and App. *Physiol. Genomics,* 20, 45-54.

ATTIEH, Z. K., MUKHOPADHYAY, C. K., SESHADRI, V., TRIPOULAS, N. A. & FOX, P. L. (1999) Ceruloplasmin Ferroxidase Activity Stimulates Cellular Iron Uptake by a Trivalent Cation-specific Transport Mechanism. *J. Biol. Chem.,* 274, 1116-1123.

BAE, B. & PERCIVAL, S. S. (1994) Copper Uptake and Intracellular Distribution During Retinoic Acid Induced Differentiation of HL-60 Cells. *J. Nutr. Biochem.,* 5, 457-461.

BALUSIKOVA, K., NEUBAUEROVA, J., DOSTALIKOVA-CIMBUROVA, M., HORAK, J. & KOVAR, J. (2009) Differing Expression of Genes Involved in Non-Transferrin Iron Transport Across Plasma Membrane in Various Cell Types Under Iron Deficiency and Excess. *Mol. Cell. Biochem.,* 321, 123-133.

BANCI, L., BERTINI, I., CANTINI, F., DELLA-MALVA, N., MIGLIARDI, M. & ROSATO, A. (2007) The Different Intermolecular Interactions of the Soluble Copper-binding Domains of the Menkes Protein, ATP7A. *J. Biol. Chem.,* 282, 23140-23146.

BANCI, L., BERTINI, I., CANTINI, F., MIGLIARDI, M., NATILE, G., NUSHI, F. & ROSATO, A. (2009) Solution Structures of the Actuator Domain of ATP7A and ATP7B, the Menkes and Wilson Disease Proteins. *Biochemistry,* 48, 7849-7855.

BANHA, J., MARQUES, L., OLIVEIRA, R., MARTINS, M. D. F., PAIXÃO, E., PEREIRA, D., MALHÓ, R., PENQUE, D. & COSTA, L. (2008) Ceruloplasmin Expression by Human Peripheral Blood Lymphocytes: A New Link Between Immunity and Iron Metabolism. *Free Radic. Biol. Med.,* 44, 483-492.

BARNES, N., BARTEE, M. Y., BRAITERMAN, L., GUPTA, A., USTIYAN, V., ZUZEL, V., KAPLAN, J. H., HUBBARD, A. L. & LUTSENKO, S. (2009) Cell-Specific Trafficking Suggests a new role for Renal ATP7B in the Intracellular Copper Storage. *Traffic,* 10, 767-779.

BARNES, N., TSIVKOVSKII, R., TSIVKOVSKAIA, N. & LUTSENKO, S. (2005) The Copper-Transporting ATPases, Menkes and Wilson Disease Proteins, Have Distinct Roles in Adult and Developing Cerebellum. *J. Biol. Chem.,* 280, 9640-9645.

BARTEE, M.Y. & LUTSENKO, S. (2007) Hepatic Copper-Transporting ATPase ATP7B: Function and Inactivation at the Molecular and Cellular. *Biometals*, 20, 627–637.

BARTHEL, A., OSTRAKHOVITCH, E. A., WALTER, P. L., KAMPKOTTER, A. & KLOTZ, L. O. (2007) Stimulation of Phosphoinositide 3-kinase/Akt Signaling by Copper and Zinc Ions: Mechanisms and Consequences. *Arch. Biochem. Biophys.,* 463, 175-182.

BAUERLY, K. A., KELLEHER, S. L. & LONNERDAL, B. (2004) Functional and Molecular Responses of Suckling Rat Pups and Human Intestinal Caco-2 Cells to Copper Treatment. *J. Nutr. Biochem.,* 15, 155-162.

BAUERLY, K. A., KELLEHER, S. L. & LONNERDAL, B. (2005) Effects of Copper Supplementation on Copper Absorption, Tissue Distribution and Copper Transporter Expression in an Infant Rat Model. *Am. J. Physiol. Gastrointest. Liver Physiol.,* 288, 1007-1014.

BELLINGHAM, S. A., LAHIRI, D. K., MALONEY, B., FONTAINE, S. L., MULTHAUP, G. & CAMAKARIS, J. (2004) Copper Depletion Down-regulates Expression of the Alzheimer's Disease Amyloid-β Precursor Protein Gene. *J. Biol. Chem.,* 279, 20378-20386.

BERTINI, I. & ROSATO, A. (2008) Menkes Disease. *Cell. Mol. Life Sci.,* 65, 89-91.

BHATT, L., HORGAN, C. P. & MCCAFFREY, M. W. (2009) Knockdown of β2-Microglobulin Perturbs the Subcellular Distribution of HFE and Hepcidin. *Biochem. Biophys. Res. Commun.,* 378, 727-731.

BHATT, L., MURPHY, C., S.O'DRISCOLL, L., CARMO-FONSECA, M., MCCAFFREY, M. W. & FLEMING, J. V. (2010) N-Glycosylation is Important for the Correct Intracellular Localization of HFE and its Ability to Decrease Cell Surface Transferrin Binding. *FEBS J.,* 277, 3219-3234.

BOIVIN, S., AOUFFEN, M. H., FOURNIER, A. & MATEESCU, M.-A. (2001) Molecular Characterization of Human and Bovine Ceruloplasmin Using MALDI-TOF Mass Spectrometry. *Biochem. Biophys. Res. Commun.,* 288, 1006-1010.

BRODERIUS, M., MOSTAD, E., WENDROTH, K. & PROHASKA, J. R. (2010) Levels of Plasma Ceruloplasmin Protein are Markedly Lower Following Dietary Copper Deficiency in Rodents. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.,* 151, 473-479.

BULL, P. C., THOMAS, G. R., ROMMENS, J. M., FORBES, J. R. & COX, D. W. (1993) The Wilson Disease Gene is a Putative Copper Transporting P-Type ATPase Similar to the Menkes Gene. *Nat. Genet.,* 5, 327-337.

BULLEN, J. J., ROGERS, H. J. & LEIGH, L. (1972) Iron-binding Proteins in Milk and Resistance to Escherichia coli Infection in Infants. *Br. Med. J.,* 1, 69-75.

CALABRESE, M. F. & MIRANKER, A. D. (2009) Metal Binding Sheds Light on Mechanisms of Amyloid Assembly. *Prion,* 3, 1-4.

CAMPBELL, C. H., BROWN, R. & LINDER, M. C. (1981) Circulating Ceruloplasmin is an Important Source of Copper for Normal and Malignant Animal Cells. *Biochim. Biophys. Acta.,* 678, 27-38.

CARRICO, R. J., DEUTSCH, H. F., BEINERT, H. & ORME-JOHNSON, W. H. (1969) Some Properties of an Apoceruloplasmin-Like Protein in Human Serum. *J. Biol. Chem.,* 244, 4141-4146.

CATER, M.A., FORBES, J., LA FONTAINE, S., COX, D. & MERCER, J.F.B. (2004) Intracellular Trafficking of the Human Wilson Protein: the Role of the Six N-Terminal Metal-Binding Sites. *Biochem. J*., 380, 805–813.

CATER, M. A., LA FONTAINE, S., SHIELD, K., DEAL, Y. & MERCER, J. F. B. (2006) ATP7B Mediates Vesicular Sequestration of Copper: Insight Into Biliary Copper Excretion. *Gastroenterology,* 130, 493-506.

CERKLEWSKI, C. L. (1979) Determination of a Copper Requirement to Support Gestation and Lactation for the Female Albino Rat. *J. Nutr.,* 109, 1529-1533.

CERVEZA, P. J., MEHRBOD, F., COTTON, S. J., LOMELI, N., LINDER, M. C., FONDA, E. G. & WICKLER, S. J. (2000) Milk Ceruloplasmin and its Expression by Mammary Gland and Liver in Pigs. *Arch. Biochem. Biophys.,* 373, 451-461.

CHANG, Y. Z., QIAN, Z. M., DU, J. R., ZHU, L., XU, Y., LI, L.-Z., WANG, C.-Y., WANG, Q., GE, X. H., HO, K. P., NIU, L. & KE, Y. (2007) Ceruloplasmin Expression and its Role in Iron Transport in C6 Cells. *Neurochem. Int.,* 50, 726-733.

CHELLY, J., TUMER, Z., TONNESEN, T., PETTERSON, A., ISHIKAWA-BRUSH, Y., TOMMERUP, N., HORN, N. & MONACO, A.P. (1993) Isolation of a Candidate Gene for Menkes Disease that Encodes a Potential Heavy Metal Binding Protein. *Nat. Genet*., 3,14–19.

CHEN, H., HUANG, G., SU, T., GAO, H., ATTIEH, Z. K., MCKIE, A. T., ANDERSON, G. J. & VULPE, C. D. (2006) Decreased Hephaestin Activity in the Intestine of Copper-Deficient Mice Causes Systemic Iron Deficiency. *J. Nutr.,* 136, 1236-1241.

CHEN, H., LIU, B., LUKAS, T. J., SUYEOKA, G., WU, G. & NEUFELD, A. H. (2009) Changes in Iron Regulatory Proteins in the Aged Rodent Neural Retina. *Neurobiol. Aging,* 30, 1865-1876.

CHEN, H. H. W., YAN, J. J., CHEN, W. C., KUO, M. T., LAI, Y. H., LAI, W. W., LIU, H. S. & SU, W. C. (2012) Predictive and Prognostic Value of Human Copper Transporter 1 (hCtr1) in Patients with Stage III Non-Small-Cell Lung Cancer Receiving First-Line Platinum-Based Doublet Chemotherapy. *Lung Cancer,* 75, 228-234.

CHEN, L., DENTCHEV, T., WONG, R., HAHN, P., WEN, R., BENNETT, J. & DUNAIEF, J. L. (2003) Increased Expression of Ceruloplasmin in the Retina Following Photic Injury. *Mol. Vis.,* 9, 151-158.

CHENG, A. S. & BAYLISS, S. J. (2008) The Genetics of Hair Shaft Disorders. *J. Am. Acad. Dermatol.,* 59, 1-22.

CHIPLONKAR, S. A., AGTE, V. V., TARWADI, K. V., PAKNIKAR, K. M. & DIWATE, U. P. (2004) Micronutrient Deficiencies as Predisposing Factors for Hypertension in Lacto-Vegetarian Indian Adults. *J. Am. Coll. Nutr.,* 23, 239-247.

CHUNG, J., CHEN, C. & PAW, H. H. (2012) Heme Metabolism and Erythropoiesis. *Curr. Opin. Hematol.,* 19, 156-162.

CLEMENTE, C., RUSSO, F., CARUSO, M. G., GIANGRANDE, M., FANIZZA, G. & LEO, A. D. (1992) Ceruloplasmin Serum Level in Post-Menopausal Women Treated with Oral Estrogens Administered at Different Times. *Horm. Metab. Res.,* 24, 191-193.

COBBOLD, C., COVENTRY, J., PONNAMBALAM, S. & MONACO, A. P. (2003) The Menkes Disease ATPase (ATP7A) is Internalized via a Rac1-Regulated, Clathrin- and Caveolae-Independent Pathway. *Hum. Mol. Genet*.,12, 1523–1533.

COLLARD, K. J. (2009) Iron Homeostasis in the Neonate. *Pediatrics,* 123, 1208-1216.

COLLINS, J. F., PROHASKA, J. R. & KNUTSON, M. D. (2010) Metabolic crossroads of iron and copper. *Nutr. Rev.,* 68, 133-147.

CONLAN, D., KORULA, R. & TALLENTIRE, D. (1990) Serum Copper Levels in Elderly Patients with Femoral-Neck Fractures *Age Ageing,* 19, 212-214.

COX, D. W. & MOORE, S. D. P. (2002) Copper Transporting P-Type ATPases and Human Disease. *J. Bioenerg. Biomembr*., 34, 333-338.

COX, C., TEKNOS, T. N., BARRIOS, M., BREWER, G. J., DICK, R. D. & MERAJVER, S. D. (2001) The Role of Copper Suppression as an Antiangiogenic Strategy in Head and Neck Squamous Cell Carcinoma. *Laryngoscope,* 111, 696-701.

COYLE, P., PHILCOXA, J. C., CAREYA, L. C. & ROFEA, A. M. (2002) Metallothionein: The Multipurpose Protein. *Cell. Mol. Life Sci.,* 59, 627-647.

CULLEN, L., PRAT, L. & COX, D. (2003) Genetic Variation in the Promoter and 50 UTR of the Copper Transporter, ATP7B, in Patients with Wilson Disease. *Clin. Genet.,* 64, 429-432.

CULOTTA, V.C., KLOMP, L.W., STRAIN, J., CASARENO, R.L., KREMS, B. & GITLIN, J.D. (1997) The Copper Chaperone for Superoxide Dismutase. *J. Biol. Chem*., 272, 23469–23472.

DANKS, D.M. (1988) The Mild Form of Menkes Disease: Progress Report on the Original Case. *Am. J. Med. Genet.,* 30, 859–864.

DANKS, D.M. (1995) *Disorders of copper transport*. In: SCRIVER, C.R., BEAUDET, A.L., SLY, W.S. & VALLE, D. *The metabolic and molecularbasis of inherited disease, 7th edn*. McGraw-Hill, New York.

DANKS, D.M., CAMPBELL, P.E., STEVENS, B.J., MAYNE, V., CARTWRIGHT, E. (1972) Menkes's Kinky Hair Syndrome. An Inherited Defect in Copper Absorption with Widespread Effects. *Pediatrics*, 50,188-201.

DAS, D., TAPRYAL, N., GOSWAMI, S. K., FOX, P. L. & MUKHOPADHYAY, C. K. (2007) Regulation of Ceruloplasmin in Human Hepatic Cells by Redox Active Copper: Identification of a Novel AP-1 Site in the Ceruloplasmin Gene. *Biochem. J.,* 402, 135-141.

DAVIDSON, L. A. & LONNERDAL, B. (1988) Specific Binding of Lactoferrin to Brush-Border Membrane: Ontogeny and Effect of Glycan Chain. *Am. J. Physiol.,* 254, 580-585.

DAVIDSON, L. A., MCORMOND, S. L. & HARRIS, E. D. (1994) Characterization of a Particulate Pathway for Copper in K562 Cells. *Biochim. Biophys. Acta.,* 1221, 1-6.

DE BIE, P., MULLER, P., WIJMENGA, C. & KLOMP, L. W. J. (2007) Molecular Pathogenesis of Wilson and Menkes Disease: Correlation of Mutations With Molecular Defects and Disease Phenotypes. *J. Med. Genet.,* 44, 673-688.

DE DOMENICO, I., WARD, D. M., DI PATTI, M. C. B., JEONG, S. Y., DAVID, S., MUSCI, G. & KAPLAN, J. (2007) Ferroxidase Activity is Required for the Stability of cell Surface Ferroportin in Cells Expressing GPI-Ceruloplasmin. *EMBO J.,* 26, 2823.

DE FEO, C. J., ALLER, S. G., SILUVAI, G. S., BLACKBURN, N. J. & UNGER, V. M. (2009) Three-Dimensional Structure of the Human Copper Transporter hCTR1. *Proc. Natl. Acad. Sci. U.S.A.,* 106, 4237-4242.

DE LORENZI, E., COLOMBO, R., SABELLA, S., CORLIN, D. B. & HEEGAARD, N. H. H. (2008) The Influence of Cu2+ on the Unfolding and Refolding of Intact and Proteolytically Processed β2-Microglobulin. *Electrophoresis,* 29, 1734-1740.

DENG, N.-J., YAN, L., SINGH, D. & CIEPLAK, P. (2006) Molecular Basis for the Cu21 Binding-Induced Destabilization of β2-Microglobulin Revealed by Molecular Dynamics Simulation. *Biophys. J.,* 90, 3865-3879.

DI PATTI, M. C. B., MAIO, N., RIZZO, G., DE FRANCESCO, G., PERSICHINI, T., COLASANTI, M., POLTICELLI, F. & MUSCI, G. (2009) Dominant Mutants of Ceruloplasmin Impair the Copper Loading Machinery in Aceruloplasminemia. *J. Biol. Chem.,* 284, 4545-4554.

DONLEY, S. A., ILAGAN, B. J., RIM, H. & LINDER, M. C. (2002) Copper Transport to Mammary Gland and Milk During Lactation in Rats. *Am. J. Physiol. Endocrinol. Metab.,* 283, 667–675.

DOREA, J. G. (2000) Iron and copper in human milk. *Nutrition,* 16, 209-220.

EISSES, J. F., CHI, Y. & KAPLAN, J. H. (2005) Stable Plasma Membrane Levels of hCTR1 Mediate Cellular Copper Uptake. *J. Biol. Chem.,* 280, 9635-9639.

EISSES, J. F. & KAPLAN, J. H. (2002) Molecular Characterization of hCTR1, the Human Copper Uptake Protein. *J. Biol. Chem.,* 277, 29162-29171.

EISSES, J. F. & KAPLAN, J. H. (2005) The Mechanism of Copper Uptake Mediated by Human CTR1. *J. Biol. Chem.,* 280, 37159-37168.

ENNS, C. A. (2001) Pumping iron: the Strange Partnership of the Hemochromatosis Protein, a Class I MHC Homolog, with the Transferrin Receptor. *Traffic,* 2, 167-174.

EVANS, J. L. & ABRAHAM, P. A. (1973) Anemia, Iron Storage and Ceruloplasmin in Copper Nutrition in the Growing Rat. *J. Nutr.,* 103, 196-201.

FERRUZZA, S., SAMBUY, Y., CIRIOLO, M. R., MARTINO, A. D., SANTARONI, P., ROTILIO, G. & SCARINO, M. L. (2000) Copper Uptake and Intracellular Distribution in the Human Intestinal Caco-2 Cell Line. *Biometals,* 13, 179-185.

FITCH, C. A., SONG, Y. & LEVENSON, C. W. (1999) Developmental Regulation of Hepatic Ceruloplasmin mRNA and Serum Activity by Exogenous Thyroxine and Dexamethasone. *Proc. Soc. Exp. Biol. Med.,* 221, 27-31.

FORBES, J. R. & COX, D.W. (1998) Functional Characterization of Missense Mutations in ATP7B: Wilson Disease Mutation or Normal Variant? *Am. J. Hum. Genet.*, 63, 1663–1674.

FORBES, J. R. & COX, D. W. (2000) Copper-Dependent Trafficking of Wilson Disease Mutant ATP7B Proteins. *Hum. Mol. Genet*., 9, 1927–1935.

FORTNA, R. R., WATSON, H. A. & NYQUIST, S. E. (1999) Glycosyl Phosphatidylinositol-Anchored Ceruloplasmin is Expressed by Rat Sertoli Cells and is Concentrated in Detergent-Insoluble Membrane Fractions. *Biol. Reprod.,* 61, 1042-1049.

FOSSET, C., DANZEISEN, R., GAMBLING, L., MCGAWB, B. A. & MCARDLE, H. J. (2009) Cu loading Alters Expression of Non-IRE Regulated, but not IRE Regulated, Fe Dependent Proteins in HepG2 Cells. *J. Inorg. Biochem.,* 103, 709-716.

FRAZER, D. M., DARSHAN, D. & ANDERSON, G. J. (2011) Intestinal iron absorption during suckling in mammals. *Biometals,* 24, 567-574.

FERREIRA, A.M., CIRIOLO, M.R., MARCOCCI. L. & ROTILIO. G. (1993) Copper (I) Transfer into Metallothionein Mediated by Glutathione. *Biochem. J.,* 292, 673–676.

FREEDMAN, J.H., CIRIOLO, M.R., & PEISACH, J. (1989) The Role of Glutathione in Copper Metabolism and Toxicity. *J. Biol. Chem.*, 264, 5598–5605.

FREEDMAN, J.H. & PEISACH. J. (1989) Intracellular Copper Transport in Cultured Hepatoma Cells. *Biochem. Biophys. Res. Commun.*, 164, 134–140.

FRIEL, J. K., ANDREWS, W. L., JACKSON, S. E., LONGERICH, H. P., MERCER, C., MCDONALD, A., DAWSON, B. & SUTRADHAR, B. (1999) Elemental Composition of Human Milk from Mothers of Premature and Full-Term Infants During the First 3 Months of Lactation. *Biol. Trace Elem. Res.,* 67, 225-247.

GAMBLING, L., DUNFORD, S. & MCARDLE, H. J. (2004) Iron Deficiency in the Pregnant Rat has Differential Effects on Maternal and Fetal Copper Levels. *J. Nutr. Biochem.,* 15, 366-372.

GEDDES, D. T. (2007) Inside the Lactating Breast: The Latest Anatomy Research. *J. Midwifery Womens Health,* 52, 566-563.

GEETHA, A., SARANYA, P., JEYACHRISTY, S. A., SURENDRAN, R. & SUNDARAM, A. (2009) Relevance of Non-Ceruloplasmin Copper to Oxidative Stress in Patients with Hepatocellular Carcinoma. *Biol. Trace Elem. Res.,* 130, 229-240.

GIRAUD, P., MALTÈSE, J.Y., BOUDOURESQUE, F., SALERS, P., OUAFIK, L., RENARD, M., PELEN, F. & OLIVER, C. (1992) Peptidylglycine Alpha-Amidating Monooxygenase Activity and TRH and CRF Biosynthesis. Role of Copper. *Biol. Trace Elem. Res*., 32, 293-301.

GITLIN, J. D. (1998) Aceruloplasminemia. *Pediatr. Res.,* 44, 271-276.

GITLIN, D. & JANEWAY, C. A. (1960) Turnover of the Copper and Protein Moieties of Ceruloplasmin. *Nature*, 185, 693.

GITLIN, J. D., SCHROEDER, J. J., LEE-AMBROSE, L. M. & COUSINS, R. J. (1992) Mechanisms of Caeruloplasmin Biosynthesis in Normal and Copper-Deficient Rats. *Biochem. J.,* 282, 835-839.

GLERUM, D.M., SHTANKO, A. & TZAGOLOFF, A. (1996) Characterization of COX17, a Yeast Gene Involved in Copper Metabolism and Assembly of Cytochrome Oxidase. *J. Biol. Chem*., 271, 14504–14509.

GOLDSTEIN, I. M., KAPLAN, H. B., EDELSON, H. S. & WEISSMANN, G. (1979) Ceruloplasmin. A Scavenger of Superoxide Anion Radicals. *J. Biol. Chem.,* 254, 4040-4045.

GONZÁLEZ-CHÁVEZ, S. A., ARÉVALO-GALLEGOS, S. & RASCÓN-CRUZ, Q. (2009) Lactoferrin: Structure, Function and Applications. *Int. J. Antimicrob. Agents,* 33, 301e1-301e8.

GOOD, M. & VASAK, M. (1986) Iron(II)-Substituted Metallothionein: Evidence for the Existence of Iron-Thiolate Clusters. *Biochemistry*, 25, 8353–8356.

GREENOUGH, M., PASE, L., VOSKOBOINIK, I., PETRIS, M. J., O'BRIEN, A. W. & CAMAKARIS, J. (2004) Signals Regulating Trafficking of Menkes (MNK; ATP7A) Copper-Translocating P-type ATPase in Polarized MDCK Cells. *Am. J. Physiol., Cell Physiol.,* 287, 1463-1471.

GULLER, S., BUHIMSCHI, C. S., MA, Y. Y., HUANG, S. T. J., YANG, L., KUCZYNSKI, E., ZAMBRANO, E., LOCKWOOD, C. J. & BUHIMSCHI, I. A. (2008) Placental Expression of Ceruloplasmin in Pregnancies Complicated by Severe Preeclampsia. *Lab. Invest.,* 88, 1057-1067.

GUO, Y., NYASAE, L., BRAITERMAN, L.T. & HUBBARD, A.L. (2005) NH2-Terminal Signals in ATP7B Cu-ATPase Mediate its Cu-Dependent Anterograde Traffic in Polarized Hepatic Cells. *Am. J. Physiol. Gastrointest. Liver Physiol*., 289, 904–916.

GUO, Y., SMITH, K., LEE, J., THIELE, D. J. & PETRIS, M. J. (2004a) Identification of Methionine-rich Clusters That Regulate Copperstimulated Endocytosis of the Human Ctr1 Copper Transporter. *J. Biol. Chem.,* 279, 17428-17433.

GUO, Y., SMITH, K. & PETRIS, M. J. (2004b) Cisplatin Stabilizes a Multimeric Complex of the Human Ctr1 Copper Transporter. *J. Biol. Chem.,* 279, 46393-46399.

HA-DUONG, N. T., EID, C., HEMADI, M. & CHAHINE, J. M. E. H. (2010) In Vitro Interaction Between Ceruloplasmin and Human Serum Transferrin. *Biochemistry,* 49, 10261-10263.

HAMZA, I., FAISST, A., PROHASKA, J., CHEN, J., GRUSS, P. & GITLIN, J. D. (2001) The Metallochaperone Atox1 Plays a Critical Role in Perinatal Copper Homeostasis. *Proc. Natl. Acad. Sci. U. S. A.,* 98, 6848-6852.

HAMZA, I., PROHASKA, J. & GITLIN, J. D. (2003) Essential Role for Atox1 in the Copper-Mediated Intracellular Trafficking of the Menkes ATPase. *Proc. Natl. Acad. Sci. U. S. A.,* 100, 1215-1220.

HARADA, M., KAWAGUCHI, T., KUMEMURA, H., TERADA, K., NINOMIYA, H., TANIGUCHI, E., HANADA. S., BABA, S., MAEYAMA, M., KOGA, H., UENO, T., FURUTA, K., SUGANUMA, T., SUGIYAMA, T. & SATA, M. (2005) The Wilson Disease Protein ATP7B Resides in the Late Endosomes with Rab7 and the Niemann-Pick C1 Protein. *Am J Pathol*, 166, 499-510.

HARDMAN, B., MANUELPILLAI, U., WALLACE, E. M., MONTY, J. F., KRAMER, D. R., KUO, Y. M., MERCER, J. F. B. & ACKLAND, M. L. (2006) Expression, Localisation and Hormone Regulation of the Human Copper Transporter hCTR1 in Placenta and Choriocarcinoma Jeg-3 Cells. *Placenta.,* 27, 968-977.

HARDMAN, B., MICHALCZYK, A., GREENOUGH, M., CAMAKARIS, J., MERCER, J. F. B. & ACKLAND, M. L. (2007) Distinct Functional Roles for the Menkes and Wilson Copper Translocating P-type ATPases in Human Placental Cells. *Cell. Physiol. Biochem.,* 20, 1073-1084.

HARDMAN, B., MICHALCZYK, A., GREENOUGH, M., CAMAKARIS, J., MERCER, J. F. B. & ACKLAND, M. L. (2007) Hormonal Regulation of the Menkes and Wilson Copper-Transporting ATPases in Human Placental Jeg-3 Cells. *Biochem. J.,* 402, 241-250.

HARRIS, Z. L., DURLEY, A. P., MAN, T. K. & GITLIN, J. D. (1999) Targeted Gene Disruption Reveals an Essential Role for Ceruloplasmin in Cellular Iron Efflux. *Proc. Natl. Acad. Sci. U. S. A.,* 96, 10812-10817.

HELLMAN, N. E. & GITLIN, J. D. (2002) Ceruloplasmin Metabolism and Function. *Annu. Rev. Nutr.,* 22, 439-458.

HELLMAN, N. E., KONO, S., MANCINI, G. M., HOOGEBOOM, A. J., JONG, G. J. D. & GITLIN, J. D. (2002a) Mechanisms of Copper Incorporation into Human Ceruloplasmin. *J. Biol. Chem.,* 277, 46632-46638.

HELLMAN, N. E., KONO, S., MIYAJIMA, H. & GITLIN, J. D. (2002b) Biochemical Analysis of a Missense Mutation in Aceruloplasminemia. *J. Biol. Chem.,* 277, 1375-1380.

HIDA, A., KOWA, H., IWATA, A., TANAKA, M., KWAK, S. & TSUJI, S. (2010) Aceruloplasminemia in a Japanese Woman with a Novel Mutation of Cp Gene: Clinical Presentations and Analysis of Genetic and Molecular Pathogenesis. *J. Neurol. Sci.,* 298, 136-139.

HILL, G. M. & LINK, J. E. (2009) Transporters in the Absorption and Utilization of Zinc and Copper. *J. Anim. Sci.,* 87, E85-E89.

HIRANO, K., OGIHARA, T., OGIHARA, H., HIROI, M., HASEGAWA, M. & TAMAI, H. (2005) Identification of Apo- and Holo-forms of Ceruloplasmin in Patients with Wilson's Disease Using Native Polyacrylamide Gel Electrophoresis. *Clin. Biochem.,* 38, 9-12.

HOCHSTRASSER, H., TOMIUK, J., WALTER, U., BEHNKE, S., SPIEGEL, J., KRUGER, R., BECKER, G., RIESS, O. & BERG, D. (2005) Functional Relevance of Ceruloplasmin Mutations in Parkinson's Disease. *FASEB J.,* 19, 1851-1853.

HOLTZMAN, N. A. & GAUMNITZ, B. M. (1970a) Identification of an Apoceruloplasmin-like Substance in the Plasma of Copper-deficient Rats. *J. Biol. Chem.,* 245, 2350-2353.

HOLTZMAN, N. A. & GAUMNITZ, B. M. (1970b) Studies on the Rate of Release and Turnover of Ceruloplasmin and Apoceruloplasmin in Rat Plasma. *J. Biol. Chem.,* 245, 2354-2358.

HOLZER, A. K. & HOWELL, S. B. (2006) The Internalization and Degradation of Human Copper Transporter 1 following Cisplatin Exposure. *Cancer Res.,* 66, 10944-10952.

HOLZER, A. K., KATANO, K., KLOMP, L. W. J. & HOWELL, S. B. (2004a) Cisplatin Rapidly Down-Regulates its own Influx Transporter hCTR1 in Cultured Human Ovarian Carcinoma Cells. *Clin. Cancer. Res.,* 10, 6744-6749.

HOLZER, A. K., SAMIMI, G., KATANO, K., NAERDEMANN, W., LIN, X., SAFAEI, R. & HOWELL, S. B. (2004b) The Copper Influx Transporter Human Copper Transport Protein 1 Regulates the Uptake of Cisplatin in Human Ovarian Carcinoma Cells. *Mol. Pharmacol.,* 66, 817-823.

HOVEY, R. C., TROTT, J. F. & VONDERHAAR, B. K. (2002) Establishing a Framework for the Functional Mammary Gland: From Endocrinology to Morphology. *J. Mammary Gland Biol. Neoplasia.,* 7, 17-38.

HOWELL, S. B., SAFAEI, R., LARSON, C. A. & SAILOR, M. J. (2010) Copper Transporters and the Cellular Pharmacology of the Platinum-Containing Cancer Drugs. *Mol. Pharmacol.,* 77, 887-894.

HSI, G. & COX, D.W. (2004) A Comparison of the Mutation Spectra of Menkes Disease and Wilson Disease. *Hum. Genet.*, 114, 165–172.

HUANG, W. C., HAVEL, J. J., ZHAU, H. E., QIAN, W. P., LUE, H.-W., CHU, C. Y., NOMURA, T. & CHUNG, L. W. K. (2008) β2-Microglobulin Signaling Blockade Inhibited Androgen Receptor Axis and Caused Apoptosis in Human Prostate Cancer Cells. *Clin. Cancer Res.,* 14, 5341-5347.

HUANG, W. C., WU, D., XIE, Z., ZHAU, H. E., NOMURA, T., ZAYZAFOON, M., POHL, J., HSIEH, C.-L., WEITZMANN, M. N., FARACH-CARSON, M. C. & CHUNG, L. W. K. (2006) β2-Microglobulin is a Signaling and Growth-Promoting Factor for Human Prostate Cancer Bone Metastasis. *Cancer Res.,* 66, 9108-9116.

HUANG, W. C., ZHAU, H. E. & CHUNG, L. W. K. (2010) Androgen Receptor Survival Signaling is Blocked by Anti-β2-microglobulin Monoclonal Antibody via a MAPK/Lipogenic Pathway in Human Prostate Cancer Cells. *J. Biol. Chem.,* 285, 7947-7956.

HUNG, I. H., SUZUKI, M., YAMAGUCHI, Y., YUAN, D. S., KLAUSNER, R. D. & GITLIN, J. D. (1997) Biochemical Characterization of the Wilson Disease Protein and Functional Expression in the Yeast Saccharomyces Cerevisiae. *J. Biol. Chem.,* 272, 21461-21466.

HUSSAIN, F., RODRIGUEZ-GRANILLO, A. & WITTUNG-STAFSHEDE, P. (2009) Lysine-60 in Copper Chaperone Atox1 Plays an Essential Role in Adduct Formation with a Target Wilson Disease Domain. *J. Am. Chem. Soc.,* 131, 16371-16373.

HUSTER, D. & LUTSENKO, S. (2007) Wilson Disease: Not Just a Copper Disorder. Analysis of a Wilson Disease Model Demonstrates the Link Between Copper and Lipid Metabolism. *Mol. Biosyst.,* 3, 816-824.

ITOH, S., KIM, H. W., NAKAGAWA, O., OZUMI, K., LESSNER, S. M., AOKI, H., AKRAM, K., MCKINNEY, R. D., USHIO-FUKAI, M. & FUKAI, T. (2008) Novel Role of Antioxidant-1 (Atox1) as a Copper-Dependent Transcription Factor Involved in Cell Proliferation. *J. Biol. Chem.,* 283, 9157-9167.

JAEGER, J. L., SHIMIZU, N. & GITLIN, J. D. (1991) Tissue-Specific Ceruloplasmin Gene Expression in the Mammary Gland. *Biochem. J.,* 280, 671-677.

JANDIAL, D. D., FARSHCHI-HEYDARI, S., LARSON, C. A., ELLIOTT, G. I., WRASIDLO, W. J. & HOWELL, S. B. (2009) Enhanced Delivery of Cisplatin to Intraperitoneal Ovarian CarcinomasMediated by the Effects of Bortezomib on the Human Copper Transporter 1. *Clin. Cancer. Res.,* 15, 553-560.

JENEY, V., ITOH, S., WENDT, M., GRADEK, Q., USHIO-FUKAI, M., HARRISON, D. G. & FUKAI, T. (2005) Role of Antioxidant-1 in Extracellular Superoxide Dismutase Function and Expression. *Circ. Res.,* 96, 723-729.

JENKINS, K. J. & HIDIROGLOU, M. (1989) Tolerance of the Calf for Excess Copper in Milk Replacer. *J. Dairy Sci.,* 72, 150-156.

JEONG, S. Y. & DAVID, S. (2003) Glycosylphosphatidylinositol-Anchored Ceruloplasmin Is Required for Iron Efflux from Cells in the Central Nervous System. *J. Biol. Chem.,* 278, 27144.

JOSSON, S., NOMURA, T., LIN, J.-T., HUANG, W.-C., WU, D., ZHAU, H. E., ZAYZAFOON, M., WEIZMANN, M. N., GURURAJAN, M. & CHUNG, L. W. K. (2011) β2-Microglobulin Induces Epithelial to Mesenchymal Transition and Confers Cancer Lethality and Bone Metastasis in Human Cancer Cells. *Cancer Res.,* 71, 2600-2610.

KAGAN, H.M. & LI, W. (2003) Lysyl Oxidase: Properties, Specificity, and Biological Roles Inside and Outside of the Cell. *J. Cell Biochem.,* 88, 660-672.

KALER, S.G., GALLO, L.K., PROUD, V.K., PERCY, A.K., MARK, Y., SEGAL, N.A., GOLDSTEIN, D.S., HOLMES, C.S. & GAHL, W.A. (1994) Occipital Horn Syndrome and a Mild Menkes Phenotype Associated with Splice Site Mutations at the MNK Locus. *Nat. Genet.,* 8, 195–202.

KARIN, M. (1996) The Regulation of AP-1 Activity by Mitogen-Activated Protein Kinases. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.,* 351, 127-134.

KARTHIKEYAN, R., MANIVASAGAM, T., ANANTHARAMAN, P., BALASUBRAMANIAN, T. & SOMASUNDARAM, S. T. (2011) Chemopreventive Effect of Padina Boergesenii Extracts on Ferric Nitrilotriacetate (Fe-NTA)-Induced Oxidative Damage in Wistar Rats. *J. Appl. Phycol.,* 23, 257-263.

KAWADA, E., MORIDAIRA, K., ITOH, K., HOSHINO, A., TAMURA, J. I. & MORITA, T. (2006) In Long-Term Bedridden Elderly Patients with Dietary Copper Deficiency, Biochemical Markers of Bone Resorption Are Increased with Copper Supplementation during 12 Weeks. *Ann. Nutr. Metab.,* 50, 420-424.

KAWAKAMI, H., DOSAKO, S. & LONNERDAL, B. (1990) Iron Uptake from Transferrin and Lactoferrin by Rat Intestinal Brush-Border Membrane Vesicles. *Am. J. Physiol.,* 258, 535-541.

KAWAKAMI, H. & LONNERDAL, B. (1991) Isolation and Function of a Receptor for Human Lactoferrin in Human Fetal Intestinal Brush-Border Membranes. *Am. J. Physiol.,* 261, 841-846.

KE, B. X., LLANOS, R. M. & MERCER, J. F. B. (2008) ATP7A Transgenic and Nontransgenic Mice are Resistant to High Copper Exposure. *J. Nutr.,* 138, 693-697.

KE, B. X., LLANOS, R. M., WRIGHT, M., DEAL, Y. & MERCER, J. F. B. (2006) Alteration of Copper Physiology in Mice Overexpressing the Human Menkes Protein ATP7A. *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* 290, 1460-1467.

KEEN, C. L., LONNERDAL, B., CLEGG, M. S., HURLEY, L. S., MORRIS, J. G., ROGERS, Q. R. & RUCKER, R. B. (1982) Developmental Changes in Composition of Cats' Milk: Trace Elements, Minerals, Protein, Carbohydrate and Fat1. *J. Nutr.,* 112, 1763-1769.

KELLEHER, S. L. & LONNERDAL, B. (2003) Marginal Maternal Zn Intake in Rats Alters Mammary Gland Cu Transporter Levels and Milk Cu Concentration and Affects Neonatal Cu Metabolism. *J. Nutr.,* 133, 2141-2148.

KELLEHER, S. L. & LONNERDAL, B. (2006) Mammary Gland Copper Transport is Stimulated by Prolactin Through Alterations in Ctr1 and Atp7A Localization. *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* 291, 1181-1191.

KIM, B. E., SMITH, K. & PETRIS, M. J. (2003) A Copper Treatable Menkes Disease Mutation Associated with Defective Trafficking of a Functional Menkes Copper ATPase. *J. Med. Genet.*, 40, 290–295.

KIM, H., SON, H. Y., BAILEY, S. M. & LEE, J. (2009) Deletion of Hepatic Ctr1 Reveals its Function in Copper Acquisition and Compensatory Mechanisms for Copper Homeostasis. *Am. J. Physiol. Gastrointest. Liver Physiol.,* 296, 356-364.

KIRCHMAN, P. A. & BOTTA, G. (2007) Copper Supplementation Increases Yeast Life Span Under Conditions Requiring Respiratory Metabolism. *Mech. Ageing Dev.,* 128, 187-195.

KLEVAY, L. M. (1998) Lack of a Recommended Dietary Allowance for Copper may be Hazardous to Your Health. *J. Am. Coll. Nutr.,* 17, 322-326.

KLEVAY, L. M. (2011) Is the Western Diet Adequate in Copper? *J. Trace. Elem. Med. Biol. ,* 25, 204-212.

KLOMP, A. E. M., JUIJN, J. A., GUN, L. T. M. V. D., BERG, I. E. T. V. D., BERGER, R. & KLOMP, L. W. J. (2003) The N-Terminus of the Human Copper Transporter 1 (hCTR1) is Localized Extracellularly, and Interacts with Itself. *Biochem. J.,* 370, 881-889.

KLOMP, A. E. M., TOPS, B. B. J., BERG, I. E. T. V. D., BERGER, R. & KLOMP, L. W. J. (2002) Biochemical Characterization and Subcellular Localization of Human Copper Transporter 1 (hCTR1). *Biochem. J.,* 364, 497-505.

KLOMP, L.W., LIN, S.J., YUAN, D.S., KLAUSNER, R.D., CULOTTA, V.C. & GITLIN, J.D. (2007) Identification and Functional Expression of HAH1,

a Novel Human Gene Involved in Copper Homeostasis. *J. Biol. Chem*., 272, 9221–9226.

KLUGER, H. M., KLUGER, Y., GILMORE-HEBERT, M., DIVITO, K., CHANG, J. T., RODOV, S., MIRONENKO, O., KACINSKI, B. M., PERKINS, A. S. & SAPI, E. (2004) cDNA Microarray Analysis of Invasive and Tumorigenic Phenotypes in a Breast Cancer Model. *Lab. Invest.,* 84, 320-331.

KOJIMAHARA, N., NAKABAYASHI, H., IKEDA, T., MORI, M., SHIKATA, T. & ESUMI, M. (1993) Abnormal Accumulation of Apo-Ceruloplasmin in a Rat Model of Wilson's Disease. *Int. Hepatol. Commun.,* 1, 228-232.

KODAMA, H., MURATA, Y. & KOBAYASHI, M. (1999) Clinical Manifestations and Treatment of Menkes Disease and its Variants. *Pediatr. Int.,* 41, 423–429.

KOMADA, H., FUJISAWA, C. & BHADHPRASIT, W. (2012) Inherited Copper Transport Disorders: Biochemical Mechanisms, Diagnosis, and Treatment. *Curr. Drug Metab*., 13, 237-250.

KONO, S., SUZUKI, H., ODA, T., MIYAJIMA, H., TAKAHASHI, Y., SHIRAKAWA, K., ISHIKAWA, K. & KITAGAWA, M. (2006a) Biochemical Features of Ceruloplasmin Gene Mutations Linked to Aceruloplasminemia. *Neuromolecular Med.,* 8, 361-374.

KONO, S., SUZUKI, H., ODA, T., SHIRAKAWA, K., TAKAHASHI, Y., KITAGAWA, M. & MIYAJIMA, H. (2007) Cys-881 is Essential for the Trafficking and Secretion of Truncated Mutant Ceruloplasmin in Aceruloplasminemia. *J. Hepatol.,* 47, 844-850.

KONO, S., SUZUKI, H., TAKAHASHI, K., TAKAHASHI, Y., SHIRAKAWA, K., MURAKAWA, Y., YAMAGUCHI, S. & MIYAJIMA, H. (2006b) Hepatic Iron Overload Associated With a Decreased Serum Ceruloplasmin Level in a Novel Clinical Type of Aceruloplasminemia. *Gastroenterology,* 131, 240-245.

KONO, S., YOSHIDA, K., TOMOSUGI, N., TERADA, T., HAMAYA, Y., KANAOKA, S. & MIYAJIMA, H. (2010) Biological Effects of Mutant Ceruloplasmin on Hepcidin-Mediated Internalization of Ferroportin. *Biochim. Biophys. Acta.,* 1802, 968-975.

KUO, Y.M., GYBINA, A.A., PYATSKOWIT, J.W., GITSCHIER, J. & PROHASKA, J.R. (2006) Copper Transport Protein (Ctr1) Levels in Mice are Tissue Specific and Dependent on Copper Status. *J. Nutr.,* 136, 21–26.

KUO, Y.-M., ZHOU, B., COSCO, D. & GITSCHIER, J. (2001) The Copper Transporter CTR1 Provides an Essential Function in Mammalian Embryonic Development. *Proc. Natl. Acad. Sci. U. S. A.,* 98, 6836-6841.

LA FONTAINE, S., FIRTH, S. D., CAMAKARIS, J., ENGLEZOU, A., THEOPHILOS, M. B., PETRIS, M. J., HOWIE, M., LOCKHART, P. J., GREENOUGH, M., BROOKS, H., REDDEL, R. R. & MERCER, J. F. B. (1998a) Correction of the Copper Transport Defect of Menkes

Patient Fibroblasts by Expression of the Menkes and Wilson ATPases. *J. Biol. Chem.,* 273, 31375-31380.

LA FONTAINE, S., FIRTH, S. D., LOCKHART, P.J., BROOKS, H., PARTON, R.G., CAMAKARIS, J. & MERCER, J. F. B. (1998b) Functional Analysis and Intracellular Localization of the Human Menkes Protein (MNK) Stably Expressed from a cDNA Construct in Chinese Hamster Ovary Cells (CHO-K1). *Hum. Mol. Genet.*, 7, 1293–1300.

LA FONTAINE, S. & MERCER, J. F. B. (2007) Trafficking of the Copper-ATPases, ATP7A and ATP7B: Role in Copper Homeostasis. *Arch. Biochem. Biophys.,* 463, 149-167.

LAMOTE, I., MEYER, E., MASSART-LEËN, A. M. & BURVENICH, C. (2004) Sex Steroids and Growth Factors in the Regulation of Mammary Gland Proliferation, Differentiation, and Involution. *Steroids,* 69, 145-159.

LANE, C., PETRIS, M.J., BENMERAH, A., GREENOUGH, M. & CAMAKARIS, J. (2004) Studies on Endocytic Mechanisms of the Menkes Copper-Translocating P-Type ATPase (ATP7A; MNK). Endocytosis of the Menkes Protein. *Biometals*, 17, 87–98.

LANIGAN, F., O'CONNOR, D., MARTIN, F. & GALLAGHER, W. M. (2007) Molecular Links Between Mammary Gland Development and Breast Cancer. *Cell. Mol. Life Sci.,* 64, 3161-3184.

LEE, C. M., LO, H. W., SHAO, R.-P., WANG, S.-C., XIA, W., GERSHENSON, D. M. & HUNG, M.-C. (2004) Selective Activation of Ceruloplasmin Promoter in Ovarian Tumors: Potential use for Gene Therapy. *Cancer Res.,* 64, 1788-1793.

LEE, J., PENA, M. M. O., NOSE, Y. & THIELE, D. J. (2002a) Biochemical Characterization of the Human Copper Transporter Ctr1. *J. Biol. Chem.,* 277, 4380-4387.

LEE, J., PETRIS, M. J. & THIELE, D. J. (2002b) Characterization of Mouse Embryonic Cells Deficient in the Ctr1 High Affinity Copper Transporter. *J. Biol. Chem.,* 277, 40253-40259.

LEE, J., PROHASKA, J. R. & THIELE, D. J. (2001) Essential Role for Mammalian Copper Transporter Ctr1 in Copper Homeostasis and Embryonic Development. *Proc. Natl. Acad. Sci. U. S. A.,* 98, 6842-6847.

LEE, S. H., LANCEY, R., MONTASER, A., MADANI, N. & LINDER, M. C. (1993) Ceruloplasmin and Copper Transport During the Latter Part of Gestation in the Rat. *Proc. Soc. Exp. Biol. Med.,* 203, 428-439.

LEONG, W. I. & LONNERDAL, B. (2005) Iron Transporters in Rat Mammary Gland: Effects of Different Stages of Lactation and Maternal Iron Status. *Am. J. Clin. Nutr.,* 81, 445-453.

LIN, S.J., PUFAHL, R.A., DANCIS, A., O'HALLORAN, T.V. & CULOTTA, V.C. (1997) A Role for the Saccharomyces Cerevisiae ATX1 Gene in Copper Trafficking and Iron Transport. *J. Biol. Chem.,* 272, 9215–9220.

LINDER, M. C. (1991) *Biochemistry of Copper,* New York, Plenum Press.

LINDER, M. C. (2002) *Biochemistry and Molecular Biology of Copper in Mammals,* Totowa, NJ, Humana Press.

LINDER, M. C. & HAZEGH-AZAM, M. (1996) Copper Biochemistry and Molecular Biology. *Am. J. Clin. Nutr.,* 63, 797S-811S.

LINDER, M. C., WOOTEN, L., CERVEZA, P., COTTON, S., SHULZE, R. & LOMELI, N. (1998) Copper Transport. *Am. J. Clin. Nutr.,* 67, 965S-971S.

LIU, J., SITARAM, A. & BURD, C. G. (2007) Regulation of Copper-Dependent Endocytosis and Vacuolar Degradation of the Yeast Copper Transporter, Ctr1p, by the Rsp5 Ubiquitin Ligase. *Traffic,* 8**,** 1375-1384.

LLANOS, R. M., MICHALCZYK, A. A., FREESTONE, D. J., CURRIE, S., LINDER, M. C., ACKLAND, M. L. & MERCER, J. F. B. (2008) Copper Transport During Lactation in Transgenic Mice Expressing the Human ATP7A Protein. *Biochem. Biophys. Res. Commun.,* 372, 613-617.

LOCKHART, P. J. & MERCER, J. F. B. (1999) Cloning and Expression Analysis of the Sheep Ceruloplasmin cDNA. *Gene.,* 236, 251-257.

LONNERDAL, B. (1996) Bioavailability of Copper. *Am. J. Clin. Nutr.,* 63, 821-829.

LUTSENKO, S., BARNES, N. L., BARTEE, M. Y. & DMITRIEV, O. Y. (2007a) Function and Regulation of Human Copper-Transporting ATPases. *Physiol. Rev.,* 87, 1011-1046.

LUTSENKO, S. & KAPLAN, J. H. (1995) Organization of P-type ATPases: Significance of Structural Diversity. *Biochemistry* 34, 15607–15613.

LUTSENKO, S., LESHANE, E. S. & SHINDE, U. (2007b) Biochemical basis of regulation of human copper-transporting ATPases. *Arch. Biochem. Biophys.,* 463, 134-148.

MAIA, P. A., FIGUEIREDO, R. C. B., ANASTÁCIO, A. S., SILVEIRA, C. L. P. D. & DONANGELO, C. M. (2007) Zinc and Copper Metabolism in Pregnancy and Lactation of Adolescent Women. *Nutrition,* 23, 248-253.

MAK, C. M. & LAM, C. W. (2008) Diagnis of Wilson's Disease: A Comprehensive Review. *Crit. Rev. Clin. Lab. Sci.,* 45, 263-290.

MANI, K., CHENG, F., HAVSMARK, B., DAVID, S. & FRANSSON, L.-Å. (2004) Involvement of Glycosylphosphatidylinositol-Linked Ceruloplasmin in the Copper/Zinc-Nitric Oxide-Dependent Degradation of Glypican-1 Heparan Sulfate in Rat C6 Glioma Cells. *J. Biol. Chem.,* 279, 12918-12923.

MARGOSHES, M. & VALLEE, B. L. (1957) A Cadmium Protein from Equine Kidney Cortex. *J. Am. Chem. Soc*., 79, 4813–4814.

MARYON, E. B., MOLLOY, S. A. & KAPLAN, J. H. (2007) O-Linked Glycosylation at Threonine 27 Protects the Copper Transporter hCTR1 from Proteolytic Cleavage in Mammalian Cells. *J. Biol. Chem.,* 282, 20376-20387.

MASTERS, B. A., QUAIFE, C. J., ERICKSON, J. C., KELLY, E. J., FROELICK, G. J., ZAMBROWICZ, B. P., BRINSTER, R. L. & PALMITER, R. D. (1994) Metallothionein III is Expressed in Neurons that Sequester Zinc in Synaptic Vesicles. *J. Neurosci*., 14, 5844–5857.

MATOBA, Y., KUMAGAI, T., YAMAMOTO, A., YOSHITSU, H. & SUGIYAMA, M. (2006) Crystallographic Evidence that the Dinuclear Copper Center of Tyrosinase is Flexible During Catalysis. *J. Biol. Chem*., 281, 8981-8990.

MATSUDA, I., PEARSON, T. & HOLTZMAN, N. A. (1974) Determination of Apoceruloplasmin by Radioimmunoassay in Nutritional Copper Deficiency, Menkes' Kinky Hair Syndrome, Wilson's Disease, and Umbilical Cord Blood. *Pediatr. Res.,* 8, 821-824.

MATTIE, M. D., MCELWEE, M. K. & FREEDMAN, J. H. (2008) Mechanism of Copper Activated Transcription: Activation of AP-1, and the JNK/SAPK and p38 Signal Transduction Pathways. *J. Mol. Biol.,* 383, 1008-1018.

MAYLE, K. M., LE, A. M. & KAMEI, D. T. (2012) The Intracellular Trafficking Pathway of Transferrin. *Biochim. Biophys. Acta.,* 1820, 264-281.

MAZUMDER, B. & FOX, P. L. (1999) Delayed Translational Silencing of Ceruloplasmin Transcript in Gamma Interferon-Activated U937 Monocytic Cells: Role of the 39 Untranslated Region. *Mol. Cell. Biol.,* 19, 6898-6905.

MCARDLE, H. J., MERCER, J. F., SARGESON, A. M. & DANKS, D. M. (1990) Effects of Cellular Copper Content on Copper Uptake and Metallothionein and Ceruloplasmin mRNA Levels in Mouse Hepatocytes. *J. Nutr.,* 120, 1370-1375.

MCNEILL, A., PANDOLFO, M., KUHN, J., SHANG, H. & MIYAJIMA, H. (2008) The Neurological Presentation of Ceruloplasmin Gene Mutations. *Eur. Neurol.,* 60, 200-205.

MEHTA, R., TEMPLETON, D. M. & O'BRIEN, P. J. (2006) Mitochondrial Involvement in Genetically Determined Transition Metal Toxicity II. Copper Toxicity. *Chem. Biol. Interact.,* 163, 77-85.

MENKES. J. H., ALTER, M., STEIGLEDER, G. K., WEAKLEY, D.R. & SUNG J. H. (1962) A Sex-Linked Recessive Disorder with Retardation of Growth, Peculiar Hair, and Focal Cerebral and Cerebellar Degeneration *Pediatrics,* 29, 764-779.

MERCER, J. F. B. (2001) The Molecular Basis of Copper-Transport Diseases. *Trends Mol. Med.,* 7, 64-69.

MERCER, J.F., LIVINGSTON, J., HALL, B., PAYNTER, J.A., BEGY, C., CHANDRASEKHARAPPA. S., LOCKHART, P., GRIMES, A., BHAVE, M. & SIEMIENIAK, D. (1993) Isolation of a Partial Candidate Gene for Menkes Disease by Positional Cloning. *Nat. Genet*.,3, 20–25.

MESHITSUKA, S., ISHIZAWA, M. & NOSE, T. (1987) Uptake and Toxic Effects of Heavy Metal Ions: Interactions Among Cadmium, Copper and Zinc in Cultured Cells. *Experientia,* 43, 151-156.

MEYER, L. A., DURLEY, A. P., PROHASKA, J. R. & HARRIS, Z. L. (2001) Copper Transport and Metabolism Are Normal in Aceruloplasminemic Mice. *J. Biol. Chem.,* 276, 36857-36861.

MICHALCZYK, A., BASTOW, E., GREENOUGH, M., CAMAKARIS, J., FREESTONE, D., TAYLOR, P., LINDER, M., MERCER, J. & ACKLAND, M. L. (2008) ATP7B Expression in Human Breast Epithelial Cells is Mediated by Lactational Hormones. *J. Histochem. Cytochem.,* 56, 389-399.

MICHALCZYK, A. A., RIEGER, J., ALLEN, K. J., MERCER, J. F. B. & ACKLAND, M. L. (2000) Defective Localization of the Wilson Disease Protein (ATP7B) in the Mammary Gland of the Toxic Milk Mouse and the Effects of Copper Supplementation. *Biochem. J.,* 352, 565-571.

MIDDLETON, R. B. & LINDER, M. C. (1993) Synthesis and Turnover of Ceruloplasmin in Rats Treated with 17 β-Estradiol. *Arch. Biochem. Biophys.,* 302, 362-368.

MILES, A. T., HAWKSWORTH, G. M., BEATTIE, J. H. & RODILLA, V. (2000) Induction, Regulation, Degradation, and Biological Significance of Mammalian Metallothioneins. *Crit. Rev. Biochem. Mol. Biol.,* 35, 35–70.

MITTAL, B., DOROUDCHI, M. M., JEONG, S. Y., PATEL, B. N. & DAVID, S. (2003) Expression of a Membrane-Bound Form of the Ferroxidase Ceruloplasmin by Leptomeningeal Cells. *Glia,* 41, 337-346.

MIYAJIMA, H. (2003) Aceruloplasminemia, an Iron Metabolic Disorder. *Neuropathology,* 23, 345-350.

MIYAJIMA, H., NISHIMURA, Y., MIZOGUCHI, K., SAKAMOTO, M., SHIMIZU, T. & HONDA, N. (1987) Familial Apoceruloplasmin Deficiency Associated with Blepharospasm and Retinal Degeneration. *Neurology,* 37, 761-767.

MOLLOY, S. A. & KAPLAN, J. H. (2009) Copper-dependent Recycling of hCTR1, the Human High Affinity Copper Transporter. *J. Biol. Chem.,* 284, 29704-29713.

MONAGHAN, P., WHITEHEAD, R. H., PERUSINGHE, N. & O'HARE, M. J. (1985) An Immunocytochemical and Ultrastructural Study of Heterogeneity in the Human Breast Carcinoma Cell Line PMC42. *Cancer Res.,* 45, 5088-5097.

MONTY, J.-F., LLANOS, R. M., MERCER, J. F. B. & KRAMER, D. R. (2005) Copper Exposure Induces Trafficking of the Menkes Protein in Intestinal Epithelium of ATP7A Transgenic Mice. *J. Nutr.,* 135, 2762-2766.

MORELOCK, M. M., CORMIER, T. A. & TOLMAN, G. L. (1988) Technetium Metallothioneins. *Inorg. Chem*., 27, 3137–3140.

MORIYA, M., HO, Y. H., GRANA, A., NGUYEN, L., ALVAREZ, A., JAMIL, R., ACKLAND, M. L., MICHALCZYK, A., HAMER, P., RAMOS, D., KIM, S., MERCER, J. F. B. & LINDER, M. C. (2008) Copper is Taken up Efficiently from Albumin and Alpha-2-Macroglobulin by Cultured Human Cells by More Than One Mechanism. *Am. J. Physiol. Cell Physiol.,* 295, 708-721.

MOSTAD, E. J. & PROHASKA, J. R. (2011) Glycosylphosphatidylinositol-Linked Ceruloplasmin is Expressed in Multiple Rodent Organs and is

Lower Following Dietary Copper Deficiency. *Exp. Biol. Med.,* 236, 298-308.

MUKHOPADHYAY, C. K., ATTIEH, Z. K. & FOX, P. L. (1998) Role of Ceruloplasmin in Cellular Iron Uptake. *Science,* 279, 714-717.

MUKHOPADHYAY, C. K., MAZUMDER, B. & FOX, P. L. (2000) Role of Hypoxia-inducible Factor-1 in Transcriptional Activation of Ceruloplasmin by Iron Deficiency. *J. Biol. Chem.,* 275, 21048-21054.

MULLER, P. A. J. & KLOMP, L. W. J. (2009) ATOX1: A Novel Copper-Responsive Transcription Factor in Mammals? *Int. J. Biochem. Cell Biol.,* 41, 1233-1236.

NAGARAJA, G. M., OTHMAN, M., FOX, B. P., ALSABER, R., PELLEGRINO, C. M., ZENG, Y., KHANNA, R., TAMBURINI, P., SWAROOP, A. & KANDPAL, R. P. (2006) Gene Expression Signatures and Biomarkers of Noninvasive and Invasive Breast Cancer Cells: Comprehensive Profiles by Representational Difference Analysis, Microarrays and Proteomics. *Oncogene,* 25, 2328-2338.

NEVILLE, M. C., MCFADDEN, T. B. & FORSYTH, I. (2002) Hormonal Regulation of Mammary Differentiation and Milk Secretion. *J. Mammary Gland Biol. Neoplasia,* 7, 49-66.

NGU, T. T., KRECISZ, S. & STILLMAN, M. J. (2010) Bismuth Binding Studies to the Human Metallothionein using Electrospray Mass Spectrometry. *Biochem. Biophys. Res. Commun*., 396, 206–212.

NIELSON, K. B., ATKIN, C. L. & WINGE D. R. (1985) Distinct Metal-Binding Configurations in Metallothionein. *J. Biol. Chem*., 260, 5342–5350.

NOSE, Y., KIM, B. E. & THIELE, D. J. (2006) Ctr1 Drives Intestinal Copper Absorption and is Essential for Growth, Iron Metabolism, Neonatal Cardiac Function. *Cell Metab.* 4, 235–244.

NYASAE, L., BUSTOS, R., BRAITERMAN, L., EIPPER, B. & HUBBARD, A. (2006) Dynamics of Endogenous ATP7A (Menkes protein) in Intestinal Epithelial Cells: Copper-Dependent Redistribution Between Two Intracellular Sites. *Am. J. Physiol. Gastrointest. Liver Physiol.,* 292, 1181-1194.

OSAKI, S., JOHNSON, D. A. & FRIEDEN, E. (1966) The Possible Significance of the Ferrous Oxidase Activity of Ceruloplasmin in Normal Human Serum. *J. Biol. Chem.,* 241, 2746-2751.

OWATARI, S., AKUNE, S., KOMATSU, M., IKEDA, R., FIRTH, S. D., CHE, X. F., YAMAMOTO, M., TSUJIKAWA, K., KITAZONO, M., ISHIZAWA, T., TAKEUCHI, T., AIKOU, T., MERCER, J. F. B., AKIYAMA, S. I. & FURUKAWA, T. (2007) Copper-Transporting P-Type ATPase, ATP7A, Confers Multidrug Resistance and its Expression is Related to Resistance to SN-38 in Clinical Colon Cancer. *Cancer Res.,* 67, 4860-4868.

OWEN, C. A. (1971) Metabolism of Copper 67 by the Copper-Deficient Rat. *Am. J. Physiol.,* 221, 1722-1727.

OZZELLO, L. (1971) Ultrastructure of the Human Mammary Gland. *Pathol Annu. 1971;6:1-59,* 6, 1-59.

PANG, W. W. & HARTMANN, P. E. (2007) Initiation of Human Lactation: Secretory Differentiation and Secretory Activation. *J. Mammary Gland Biol. Neoplasia,* 12, 211-221.

PASE, L., VOSKOBOINIK, I., GREENOUGH, M. & CAMAKARIS, J. (2004) Copper Stimulates Trafficking of a Distinct Pool of the Menkes Copper ATPase (ATP7A) to the Plasma Membrane and Diverts it into a Rapid Recycling Pool. *Biochem. J.,* 378, 1031-1037.

PATEL, B. N. & DAVID, S. (1997) A Novel Glycosylphosphatidylinositol-anchored Form of Ceruloplasmin is Expressed by Mammalian Astrocytes. *J. Biol. Chem.,* 272, 20185-20190.

PATEL, B. N., DUNN, R. J. & DAVID, S. (2000) Alternative RNA Splicing Generates a Glycosylphosphatidylinositolanchored Form of Ceruloplasmin in Mammalian Brain. *J. Biol. Chem.,* 275, 4305-4310.

PATEL, B. N., DUNN, R. J., JEONG, S. Y., ZHU, Q., JULIEN, J. P. & DAVID, S. (2002) Ceruloplasmin Regulates Iron Levels in the CNS and Prevents Free Radical Injury. *J. Neurosci.,* 22, 6578-6586.

PATRUTA, S. I. & HÖRL, W. H. (1999) Iron and Infection *Kidney Int. Suppl.,* 55, S125-S130.

PENA, M. M. O., LEE, J. & THIELE, D. J. (1999) A Delicate Balance: Homeostatic Control of Copper Uptake and Distribution. *J. Nutr.,* 129, 1251–1260.

PENKOWA, M., TIO, L., GIRALT, M., QUINTANA, A., MOLINERO, A., ATRIAN, S., VASÁK, M. & HIDALGO, J. (2006) Specificity and Divergence in the Neurobiologic Effects of Different Metallothioneins after Brain Injury. *J. Neurosci. Res*., 83, 974-84.

PERCIVAL, S. S. & HARRIS, E. D. (1990) Copper Transport from Ceruloplasmin: Characterization of the Cellular Uptake Mechanism. *Am. J. Physiol.,* 258, 140-146.

PERRONE, L., PALMA, L. D., TORO, R. D., GIALANELLA, G. & MORO, R. (1993) Trace Element Content of Human Milk During Lactation. *J. Trace Elem. Electrolytes Health Dis.,* 7, 245-247.

PERSICHINI, T., MAIO, N., DI PATTI, M. C. B., RIZZO, G., COLASANTI, M. & MUSCIA, G. (2010) Interleukin-1β Induces Ceruloplasmin and Ferroportin-1 Gene Expression via MAP Kinases and C/EBPβ, AP-1, and NF-κβ Activation. *Neurosci. Lett.,* 484, 133-138.

PETERSON, J., DROLET, B. A. & ESTERLY, N. B. (1998) What Syndrome is This? *Pediatr. Dermatol.,* 15, 137-139.

PETRIS, M.J., CAMAKARIS, J., GREENOUGH, M., LA FONTAINE, S., MERCER, J.F.B. (1998) A C-terminal Di-Leucine is Required for Localization of the Menkes Protein in the Trans-Golgi Network. *Hum. Mol. Genet*., 7, 2063–2071.

PETRIS, M. J. & MERCER, J. F. (1999) The Menkes Protein (ATP7A; MNK) Cycles via the Plasma Membrane Both in Basal and Elevated

Extracellular Copper Using a C-Terminal Di-Leucine Endocytic Signal. *Hum. Mol. Genet.,* 8, 2107-2115.

PETRIS, M. J., MERCER, J. F. B., CULVENOR, J. G., LOCKHART, P., GLEESON, P. A. & CAMAKARIS, J. (1996) Ligand-Regulated Transport of the Menkes Copper P-type ATPase Efflux Pump From the Golgi Apparatus to the Plasma Membrane: A Novel Mechanism of Regulated Trafficking. *EMBO J.,* 15, 6084-6095.

PETRIS, M. J., SMITH, K., LEE, J. & THIELE, D. J. (2003) Copper-stimulated Endocytosis and Degradation of the Human Copper Transporter, hCtr1. *J. Biol. Chem.,* 278, 9639-9646.

PLATONOVA, N., GUOLIKHANDANOVA, N., TSYMBALENKO, N., ZHIGULEVA, E., ZHIVULKO, T., VASIN, A., EVSUKOVA, I. & PUCHKOVA, L. (2007) Milk Ceruloplasmin is a Valuable Source of Nutrient Copper Ions for Mammalian Newborns. *J. Trace Elem. Med. Biol.,* 21, 184-193.

PROCOPIS. P., CAMAKARIS. J. & DANKS. D.M. (1981) A Mild Form of Menkes Steely Hair Syndrome. *J. Pediatr.*, 98, 97–99.

PROHASKA, J. R. (2008) Role of Copper Transporters in Copper Homeostasis. *Am. J. Clin. Nutr.,* 88, 826S-829S.

PROHASKA, J. R. & GYBINA, A. A. (2004) Intracellular Copper Transport in Mammals. *J. Nutr.,* 134, 1003-1006.

PUIG, S., LEE, J., LAU, M. & THIELE, D. J. (2002) Biochemical and Genetic Analyses of Yeast and Human High Affinity Copper Transporters Suggest a Conserved Mechanism for Copper Uptake. *J. Biol. Chem.,* 277, 26021-26030.

QIAN, Y., TIFFANY-CASTIGLIONI, E. & HARRIS, E. D. (1998) Sequence of a Menkes-type Cu-transporting ATPase from Rat C6 Glioma Cells: Comparison of the Rat Protein with Other Mammalian Cu-Transporting ATPases. *Mol. Cell. Biochem.,* 181, 49-61.

QIAN, Z. M., CHANG, Y. Z., LEUNG, G., DU, J. R., ZHU, L., WANG, Q., NIU, L., XU, Y. J., YANG, L., HO, K. P. & KE, Y. (2007) Expression of Ferroportin1, Hephaestin and Ceruloplasmin in Rat Heart. *Biochim. Biophys. Acta.,* 1772, 527-532.

QIAN, Z. M., TSOI, Y. K., KE, Y. & WONG, M. S. (2001) Ceruloplasmin Promotes Iron Uptake Rather Than Release in BT325 Cells. *Exp. Brain Res.,* 140, 369-374.

QUAIFE, C. J., FINDLEY, S. D., ERICKSON, J. C., FROELICK, G. J., KELLY, E. J., ZAMBROWICZ, B. P. & PALMITER, R. D. (1994) Induction of a New Metallothionein Isoform (MT-IV) Occurs During Differentiation of Stratified Squamous Epithelia. *Biochemistry*, 33, 7250–7259.

RANGANATHAN, P., LU, Y., JIANG, L., KIM, C. & COLLINS, J. F. (2011) Serum Ceruloplasmin (Cp) Protein Expression and Activity Increases in Iron Deficient Rats and is Further Enhanced by Higher Dietary Copper Intake. *Blood,* 118, 3146-3153.

RATHORE, K. I., KERR, B. J., REDENSEK, A., LOPEZ-VALES, R., JEONG, S. Y., PONKA, P. & DAVID, S. (2008) Ceruloplasmin Protects Injured Spinal Cord from Iron-Mediated Oxidative Damage. *J. Neurosci.,* 28, 12736-12747.

RAUCH, H. (1983) Toxic Milk, a New Mutation Affecting Copper Metabolism in the Mouse. *J. Hered.,* 74, 141-144.

RAUCH, H. (1985) Hepatic Copper in Neonatal Toxic Milk Mice. *Genetics 1985;110:S88,* 110, S88.

RAVIA, J. J., STEPHEN, R. M., GHISHAN, F. K & COLLINS J. F. (2005) Menkes Copper ATPase (Atp7a) is a Novel Metal-Responsive Gene in Rat Duodenum, Immunoreactive Protein is Present on Brush-Border and Basolateral Membrane Domains. *J. Biol. Chem*., 280, 36221–36227.

RECALCATI, S., LOCATI, M., GAMMELLA, E., INVERNIZZI, P. & CAIRO, G. (2012) Iron Levels in Polarized Macrophages: Regulation of Immunity and Autoimmunity. *Autoimmun. Rev.,* Article In Press.

RHOADS, R. E. & GRUDZIEN-NOGALSKA, E. (2007) Translational Regulation of Milk Protein Synthesis at Secretory Activation. *J. Mammary Gland Biol. Neoplasia,* 12, 283-292.

RICHARDSON, J., THOMAS, K.A., RUBIN, B.H. & RICHARDSON, D.C. (1975) Crystal Structure of Bovine Cu, Zn Superoxide Dismutase at 3 A Resolution: Chain Tracing and Metal Ligands. *Proc. Natl. Acad. Sci. U.S.A.*, 72, 1349-1353.

RILLEMA, J. A. (1994) Development of the Mammary Gland and Lactation. *Trends Endocrinol. Metab.,* 5, 149-154.

RIORDAN, S.M. & WILLIAMS, R. (2001) The Wilson's Disease Gene and Phenotypic Diversity. *J. Hepatol.* 34,165–171.

RODRIGUEZ-GRANILLO, A., CRESPO, A. & WITTUNG-STAFSHEDE, P. (2010) Interdomain Interactions Modulate Collective Dynamics of the Metal-Binding Domains in the Wilson Disease Protein. *J. Phys. Chem. B.,* 114, 1836-1848.

ROELFOSEN, H., WOLTERS, H., VAN LUYN, M. J. A., MIURA, N., KUIPERS, F. & VONK, R. J. (2000) Copper-Induced Apical Traffickingof ATP7B in Polarized Hepatoma Cells Provides a Mechanism for Biliary Copper Excretion. *Gastroenterology,* 119, 782–793.

ROWLEY, D. R., DANG, T. D., MCBRIDE, L., GERDES, M. J., LU, B. & LARSEN, M. (1995) β-2 Microglobulin is Mitogenic to PC-3 Prostatic Carcinoma Cells and Antagonistic to Transforming Growth Factor β1 Action. *Cancer Res.,* 55, 781-786.

RUSH, R.A. & GEFFEN, L.B. (1980) Dopamine Beta-Hydroxylase in Health and Disease. *Crit. Rev. Clin. Lab. Sci*. 12, 241-277.

SAARI, J. T., WOLD, L. E., DUAN, J., REN, J., CARLSON, H. L., BODE, A. M., LENTSCH, A. B., ZENG, H. & SCHUSCHKE, D. A. (2007) Cardiac Nitric Oxide Synthases are Elevated in Dietary Copper Deficiency. *J. Nutr. Biochem.,* 18, 443-448.

SABATUCCI, A., VACHETTE, P., VASILYEV, V. B., BELTRAMINI, M., SOKOLOV, A., PULINA, M., SALVATO, B., ANGELUCCI, C. B., MACCARRONE, M., COZZANI, I. & DAINESE, E. (2007) Structural Characterization of the Ceruloplasmin: Lactoferrin Complex in Solution. *J. Mol. Biol.,* 371, 1038-1046.

SAFAEI, R., MAKTABI, M. H., BLAIR, B. G., LARSON, C. A. & HOWELL, S. B. (2009) Effects of the Loss of Atox1 on the Cellular Pharmacology of Cisplatin. *J. Inorg. Biochem.,* 103, 333-341.

SALMENPERA, L., PERHEENTUPA, J., PAKARINEN, P. & SIIMES, M. A. (1986) Cu Nutrition in Infants During Prolonged Exclusive Breast-Feeding: Low Intake but Rising Serum Concentrations of Cu and Ceruloplasmin. *Am. J. Clin. Nutr.,* 43, 251-257.

SAMET, J. M., GRAVES, L. M., QUAY, J., DAILEY, L. A., DEVLIN, R. B., GHIO, A. J., WU, W., BROMBERG, P. A. & REED, W. (1998) Activation of MAPKs in Human Bronchial Epithelial Cells Exposed to Metals. *Am. J. Physiol.,* 275, 551-558.

SAMIMI, G., SAFAEI, R., KATANO, K., HOLZER, A. K., ROCHDI, M., TOMIOKA, M., GOODMAN, M. & HOWELL, S. B. (2004) Increased Expression of the Copper Efflux Transporter ATP7A Mediates Resistance to Cisplatin, Carboplatin, and Oxaliplatin in Ovarian Cancer Cells. *Clin. Cancer. Res.,* 10, 4661-4669.

SAMIMI, G., VARKI, N. M., WILCZYNSKI, S., SAFAEI, R., ALBERTS, D. S. & HOWELL, S. B. (2003) Increase in Expression of the Copper Transporter ATP7A During Platinum Drug-Based Treatment is Associated with Poor Survival in Ovarian Cancer Patients. *Clin. Cancer Res.,* 9, 5853-5859.

SÁNCHEZ, C., LÓPEZ-JURADO, M., ARANDA, P. & LLOPIS, J. (2010) Plasma Levels of Copper, Manganese and Selenium in an Adult Population in Southern Spain: Influence of Age, Obesity and Lifestyle Factors. *Sci. Total. Environ.,* 408, 1014-1020.

SARKAR, J., SESHADRI, V., TRIPOULAS, N. A., KETTERER, M. E. & FOX, P. L. (2003) Role of Ceruloplasmin in Macrophage Iron Efflux During Hypoxia. *J. Biol. Chem.,* 278, 44018-44024.

SATO, M., & BREMNER, I. (1993) Oxygen Free Radicals and Metallothionein. Free Radic. Biol. Med., 14, 325-37.

SATO, M. & GITLIN, J. D. (1991) Mechanisms of Copper Incorporation During the Biosynthesis of Human Ceruloplasmin. *J. Biol. Chem.,* 266, 5128-5134.

SCHAEFER, M., HOPKINS, R. G., FAILLA, M. L. & GITLIN, J. D. (1999a) Hepatocyte-Specific Localization and Copper-Dependent Trafficking of the Wilson's Disease Protein in the Liver. *Am. J. Physiol.,* 276, 639-646.

SCHAEFER, M., ROELOFSEN, H., WOLTERS, H., HOFMANN, W. J., MÜLLER, M., KUIPERS, F., STREMMEL, W. & VONK, R. J. (1999b) Localization of the Wilson's Disease Protein in Human Liver. *Gastroenterology,* 117, 1380-1385.

SCHEINBERG, I. H. & GITLIN, D. (1952) Deficiency of Ceruloplasmin in Patients with Hepatolenticular Degeneration. *Science*, 116, 484–485.

SCHILSKY, M. L., STOCKERT, R. J., KESNER, A., GORLA, G. R., GAGLIARDI, G. S., TERADA, K., MIURA, N. & CZAJA, M. J. (1998) Copper Resistant Human Hepatoblastoma Mutant Cell Lines Without Metallothionein Induction Overexpress ATP7B. *Hepatology,* 28, 1347-1356.

SCHRAMEL, P., MÜLLER-HÖCKER, J., MEYER, U., WEISS, M. & EIFE, R. (1988) Nutritional Copper Intoxication in Three German Infants With Severe Liver Cell Damage (Features of Indian Childhood Cirrhosis). *J. Trace Elem. Electrolytes Health Dis.,* 2, 85-89.

SESHADRI, V., FOX, P. L. & MUKHOPADHYAY, C. K. (2002) Dual Role of Insulin in Transcriptional Regulation of the Acute Phase Reactant Ceruloplasmin. *J. Biol. Chem.,* 277, 27903-27911.

SHIN, J.Y., SUH, D., KIM, J. M., CHOI, H.-G., KIM, J. A., KO, J. J., LEE, Y. B., KIM, J.-S. & OH, Y.-K. (2005) Low Molecular Weight Polyethylenimine for Efficient Transfection of Human Hematopoietic and Umbilical Cord Blood-Derived CD34+ Cells. *Biochim. Biophys. Acta.,* 1725, 377-384.

SIDHU, A., MILLER, P. J. & HOLLENBACH, A. D. (2011) FOXO1 Stimulates Ceruloplasmin Promoter Activity in Human Hepatoma Cells Treated with IL-6. *Biochem. Biophys. Res. Commun.,* 404, 963-967.

SOKOL, R.J., TWEDT, D., MCKIM, J.M., DEVEREAUX, M.W., KARRER, F.M., KAM, I., VON STEIGMAN, G., NARKEWICZ, M.R., BACON, B.R., BRITTON, R.S., & NEUSCHWANDERTETRI, B.A. (1994) Oxidant Injury to Hepatic Mitochondria in Patients with Wilson's Disease and Bedlington Terriers with Copper Toxicosis. *Gastroenterology,* 107, 1788–1798.

SOKOLOV, A. V., AGEEVA, K. V., PULINA, M. O., ZAKHAROVA, E. T. & VASILYEV, V. B. (2009) Effect of lactoferrin on oxidative features of ceruloplasmin. *Biometals,* 22, 521-529.

SOKOLOV, A. V., PULINA, M. O., AGEEVA, K. V., AYRAPETOV, M. I., BERLOV, M. N., VOLGIN, G. N., MARKOV, A. G., YABLONSKY, P. K., KOLODKIN, N. I., ZAKHAROVA, E. T. & VASILYEV, V. B. (2007) Interaction of Ceruloplasmin, Lactoferrin, and Myeloperoxidase. *Biochemistry (Mosc),* 72, 409-415.

SOKOLOV, A. V., PULINA, M. O., ZAKHAROVA, E. T., SHAVLOVSKI, M. M. & VASILYEV, V. B. (2005) Effect of Lactoferrin on the Ferroxidase Activity of Ceruloplasmin. *Biochemistry (Mosc),* 70, 1015-1019.

SOKOLOV, A. V., PULINA, M. O., ZAKHAROVA, E. T., SUSOROVA, A. S., RUNOVA, O. L., KOLODKIN, N. I. & VASILYEV, V. B. (2006) Identification and Isolation from Breast Milk of Ceruloplasmin–Lactoferrin Complex. *Biochemistry (Mosc),* 71, 160-166.

SONG, I.S., CHEN, H. H. W., AIBA, I., HOSSAIN, A., LIANG, Z. D., KLOMP, L. W. J. & KUO, M. T. (2008) Transcription Factor Sp1 Plays an Important Role in the Regulation of Copper Homeostasis in Mammalian Cells. *Mol. Pharmacol.,* 74, 705-713.

STEIN, T., SALOMONIS, N., NUYTEN, D. S. A., VIJVER, M. J. V. D. & GUSTERSON, B. A. (2009) A Mouse Mammary Gland Involution mRNA Signature Identifies Biological Pathways Potentially Associated with Breast Cancer Metastasis. *J. Mammary Gland Biol. Neoplasia,* 14, 99-116.

STEINEBACH, O. M. & WOLTERBEEK, H. T. (1994) Effects of Copper on Rat Hepatoma HTC Cells and Primary Cultured Rat Hepatocytes. *J. Inorg. Biochem.,* 53, 27-48.

STERNLIEB, I., MORELL, A.G., TUCKER, W.D., GREENE, M.W. & SCHEINBERG, I.H. (1961) The Incorporation of Copper into Ceruloplasmin in Vivo: Studies with Copper and Copper. *J. Clin. Invest.*, 40, 1834–1840.

STERNLIEB, I., VAN DEN HAMER, C.J., MORELL, A.G., ALPERT, S., GREGORIADIS, G. & SCHEINBERG, I.H. (1973) Lysosomal Defect of Hepatic Copper Excretion in Wilson's Disease (Hepatolenticular Degeneration). *Gastroenterology*, 64, 99–105.

STRAUSAK, D., HOWIE, M. K., FIRTH, S. D., SCHLICKSUPP, A., PIPKORN, R., MULTHAUP, G. & MERCER, J. F. B. (2003) Kinetic Analysis of the Interaction of the Copper Chaperone Atox1 with the Metal Binding Sites of the Menkes Protein. *J. Biol. Chem.,* 278, 20821-20827.

STRAUSAK, D., LA FONTAINE, S., HILL, J., FIRTH, S. D., LOCKHART, P. J. & MERCER, J. F. (1999) The Role of GM*X*C*XX*C Metal Binding Sites in the Copper-induced Redistribution of the Menkes Protein *J. Biol. Chem.* 274, 11170–11177.

STURTZ, L.A., DIEKERT, K., JENSEN, L.T., LILL, R. & CULOTTA, V.C. (2001) A Fraction of Yeast Cu, Zn Superoxide Dismutase and its Metallochaperone, CCS, Localize to the Intermembrane Space of Mitochondria. A Physiological Role for SOD1 in Guarding Against Mitochondrial Oxidative Damage. J. Biol. Chem., 276, 38084-38089.

SUTHERLAND, D. E. K. & STILLMAN, M. J. (2011) The ''Magic Numbers'' of Metallothionein. *Metallomics,* 3, 444-463.

TANCHOU, V., GAS, F., URVOAS, A., COUGOULUÈGNE, F., RUAT, S., AVERSENG, O. & QUÉMÉNEUR, E. (2004) Copper-Mediated Homo-Dimerisation for the HAH1 Metallochaperone. *Biochem. Biophys. Res. Commun.,* 325, 388-394.

TANNENBAUM, M., WEISS, M. & MARX, A. J. (1969) Ultrastructure of the Human Mammary Ductule. *Cancer,* 23, 958-978.

TAPIERO, H., TOWNSEND, D. M. & TEW, K. D. (2003) Trace Elements in Human Physiology and Pathology. Copper. *Biomed. Pharmacother.,* 57, 386-398.

TAPRYAL, N., MUKHOPADHYAY, C., DAS, D., FOX, P. L. & MUKHOPADHYAY, C. K. (2009) Reactive Oxygen Species Regulate Ceruloplasmin by a Novel mRNA Decay Mechanism Involving Its 3-Untranslated Region. *J. Biol. Chem.,* 284, 1873-1883.

TAPRYAL, N., MUKHOPADHYAY, C., MISHRA, M. K., DAS, D., BISWAS, S. & MUKHOPADHYAY, C. K. (2010) Glutathione Synthesis Inhibitor

Butathione Sulfoximine Regulates Ceruloplasmin by Dual but Opposite Mechanism: Implication in Hepatic Iron Overload. *Free Radic. Biol. Med.,* 48, 1492-1500.

TCHAPARIAN, E.J., URIU-ADAMS, J. Y., KEEN, C.L., MITCHELL, A. E. & RUCKER, R. B. (2000) Lysyl Oxidase and P-ATPase-7A Expression During Embryonic Development in the Rat. *Arch. Biochem. Biophys.*, 379, 71–77.

TENNANT, J., STANSFIELD, M., YAMAJI, S., SRAI, S. K. & SHARP, P. (2002) Effects of Copper on the Expression of Metal Transporters in Human Intestinal Caco-2 Cells. *FEBS Lett.,* 527, 239-244.

TERADA, K., AIBA, N., YANG, X. L., IIDA, M., NAKAI, M., MIURA, N. & SUGIYAMA, T. (1999) Biliary Excretion of Copper in LEC Rat After Introduction of Copper Transporting P-type ATPase, ATP7B. *FEBS Lett.,* 448, 53-56.

TERADA, K., KAWARADA, Y., MIURA, N., YASUI, O., KOYAMA, K. & SUGIYAMA, T. (1995) Copper Incorporation into Ceruloplasmin in Rat Livers. *Biochim. Biophys. Acta.,* 1270, 58-62.

TERADA, K., NAKAKO, T., YANG, X. L., IIDA, M., AIBA, N., MINAMIYA, Y., NAKAI, M., SAKAKI, T., MIURA, N. & SUGIYAMA, T. (1998) Restoration of Holoceruloplasmin Synthesis in LEC Rat after Infusion of Recombinant Adenovirus Bearing WND cDNA. *J. Biol. Chem.,* 273, 1815-1820.

THIRUMOORTHY, N., SUNDER, A. S., KUMAR, K. T. M., KUMAR, M. S., GANESH, G. N. K. & CHATTERJEE, M. (2011) A Review of Metallothionein Isoforms and their Role in Pathophysiology. *World J. Surg. Oncol*., 9, 54.

THOMPSON, T., FREESTONE, D., MICHALCZYK, A. A. & ACKLAND, M. L. (2012) Copper Levels in Buccal Cells of Vineyard Workers Engaged in Various Activities. *Ann. Occup. Hyg.,* 56, 305-314.

TSUKIHARA, T., AOYAMA, H., YAMASHITA, E., TOMIZAKI, T., YAMAGUCHI, H., SHINZAWA-ITOH, K., NAKASHIMA, R., YAONO, R. & YOSHIKAWA, S. (1995) Structures of Metal Sites of Oxidized Bovine Heart Cytochrome C Oxidase at 2.8 A. *Science*. 269, 1069-1074.

TSYMBALENKO, N. V., GYULIKHANDANOVA, N. E., PLATONOVA, N. A., BABICH, V. S., EVSYUKOVA, I. I. & PUCHKOVA, L. V. (2009) Regulation of Ceruloplasmin Gene Activity in Mammary Gland Cells. *Russ. J. Genet.,* 45, 341-350.

TSYMBALENKO, N. V., PLATONOVA, N. A., PUCHKOVA, L. V., MOKSHINA, S. V., SASINA, L. K., SKVORTSOVA, N. N., MISHCHENKO, B. S., EGOROV, T. A. & GAITSKHOKI, V. S. (2000) The Identification of the Ceruloplasmin Region Interacting with the Copper Transferring Menkes ATPase. *RJBC,* 26, 520-526.

TUMER. Z., MOLLER, L. B. & HORN. N. (1999) Mutation Spectrum of ATP7A, the Gene Defective in Menkes Disease. *Adv. Exp. Med. Biol*., 448, 83–95.

TUMER. Z. & MOLLER, L. B. (2010) Menkes Disease. *Eur. J. Hum. Genet*., 18, 511-518.

TURNLUND, J. R. (1998) Human Whole-Body Copper Metabolism. *Am. J. Clin. Nutr.,* 67, 960S-964S.

TYE, S. L., GILG, A. G., TOLLIVER, L. B., WHEELER, W. G., TOOLE, B. P. & MARIA, B. L. (2008) Hyaluronan Regulates Ceruloplasmin Production by Gliomas and Their Treatment-Resistant Multipotent Progenitors. *J. Child Neurol.,* 23, 1221-1230.

UCHIDA, Y., TAKIO, K., TITANI, K., IHARA, Y. & TOMONAGA, M. (1991) The Growth Inhibitory Factor that is Deficient in the Alzheimer's Disease Brain is a 68 Amino Acid Metallothionein-like Protein. *Neuron*, 7, 337-347.

URANI, C., CALINI, V., MELCHIORETTO, P., MORAZZONI, F., CANEVALI, C. & CAMATINI, M. (2003) Different Induction of Metallothioneins and Hsp70 and Presence of the Membrane Transporter ZnT-1 in HepG2 Cells Exposed to Copper and Zinc. *Toxicol. In Vitro.,* 17, 553-559.

VAN DONGEN, E. M. W. M., KLOMP, L. W. J. & MERKX, M. (2004) Copper-Dependent Protein–Protein Interactions Studied by Yeast Two-Hybrid Analysis. *Biochem. Biophys. Res. Commun.,* 323, 789-795.

VASAK, M. & MELONI, G. (2011) Chemistry and Biology of Mammalian Metallothioneins. *J. Biol. Inorg. Chem*., 16, 1067–1078.

VECCHI, C., MONTOSI, G. & PIETRANGELO, A. (2010) Huh-7: A Human “Hemochromatotic” Cell Line. *Hepatology,* 51, 654.

VELDHUIS, N. A., GAETH, A. P., PEARSON, R. B., GABRIEL, K. & CAMAKARIS, J. (2009) The Multi-Layered Regulation of Copper Translocating P-type ATPases. *Biometals,* 22, 177-190.

VONK, W. I. M., WIJMENGA, C. & VAN DE SLUIS, B. (2008) Relevance of Animal Models for Understanding Mammalian Copper Homeostasis. *Am. J. Clin. Nutr.,* 88, 840S-845S.

VOSKOBOINIK, I., BROOKS, H., SMITH, S., SHEN, P. & CAMAKARIS, J. (1998) ATP-Dependent Copper Transport by the Menkes Protein in Membrane Vesicles Isolated from Cultured Chinese Hamster Ovary Cells *FEBS Letters*, 435, 178-182.

VOSKOBOINIK, I., STRAUSAK, D., GREENOUGH, M., BROOKS, H., PETRIS, M., SMITH, S., MERCER, J.F., & CAMAKARIS, J. (1999) Functional Analysis of the N-Terminal CXXC Metal-Binding Motifs in the Human Menkes Copper-Transporting P-Type ATPase Expressed in Cultured Mammalian Cells. *J. Biol. Chem*., 274, 22008-22012.

VULPE, C., LEVINSON, B., WHITNEY, S., PACKMAN, S. & GITSCHIER, J. (1993) Isolation of a Candidate Gene for Menkes Disease and Evidence that it Encodes a Copper-Transporting ATPase. *Nat. Genet.,* 3, 7-13.

WAGGONER, D. J., BARTNIKAS, T. B. & GITLIN, J. D. (1999) The Role of Copper in Neurodegenerative Disease. *Neurobiol. Dis.,* 6, 221-230.

WAHEED, A., GRUBB, J. H., ZHOU, X. Y., TOMATSU, S., FLEMING, R. E., COSTALDI, M. E., BRITTON, R. S., BACON, B. R. & SLY, W. S.

(2002) Regulation of Transferrin-Mediated Iron Uptake by HFE, the Protein Defective in Hereditary Hemochromatosis. *Proc. Natl. Acad. Sci. U. S. A.,* 99, 3117-3122.

WALKER, J. M., TSIVKOVSKII, R. & LUTSENKO, S. (2002) Metallochaperone Atox1 Transfers Copper to the NH2-terminal Domain of the Wilson's Disease Protein and Regulates Its Catalytic Activity. *J. Biol. Chem.,* 277, 27953-27959.

WALKER, N. I. (1999) Copper Toxicosis in an Australian Child. *Eur. J. Med. Res.,* 28, 249-251.

WALTER, P. L., KAMPKÖTTER, A., ECKERS, A., BARTHEL, A., SCHMOLL, D., SIES, H. & KLOTZ, L.-O. (2006) Modulation of FoxO Signaling in Human Hepatoma Cells by Exposure to Copper or Zinc Ions. *Arch. Biochem. Biophys.,* 454, 107-113.

WANG, T. P., QUINTANAR, L., SEVERANCE, S., SOLOMON, E. I. & KOSMAN, D. J. (2003) Targeted Suppression of the Ferroxidase and Iron Trafficking Activities of the Multicopper Oxidase Fet3p from Saccharomyces Cerevisiae. *J. Biol. Inorg. Chem.,* 8, 611-620.

WANG, T. & WEINMAN, S. A. (2004) Involvement of Chloride Channels in Hepatic Copper Metabolism: ClC-4 Promotes Copper Incorporation Into Ceruloplasmin. *Gastroenterology,* 126, 1157-1166.

WEINER, A. L. & COUSINS, R. J. (1983) Hormonally Produced Changes in Caeruloplasmin Synthesis and Secretion in Primary Cultured Rat Hepatocytes. *Biochem. J.,* 212, 297-304.

WELCH, K. D., HALL, J. O., DAVIS, T. Z. & AUST, S. D. (2007) The Effect of Copper Deficiency on the Formation of Hemosiderin in Sprague-Dawley Rats. *Biometals,* 20, 829-839.

WHITE, C., KAMBE, T., FULCHER, Y. G., SACHDEV, S. W., BUSH, A. I., FRITSCHE, K., LEE, J., QUINN, T. P. & PETRIS, M. J. (2009a) Copper Transport into the Secretory Pathway is Regulated by Oxygen in Macrophages. *J. Cell. Sci.,* 122, 1315-1321.

WHITE, C., LEE, J., KAMBE, T., FRITSCHE, K. & PETRIS, M. J. (2009b) A Role for the ATP7A Copper-transporting ATPase in Macrophage Bactericidal Activity. *J. Biol. Chem.,* 284, 33949-33956.

WHITEHEAD, R. H., BERTONCELLO, I., WEBBER, L. M. & PEDERSEN, J. S. (1983a) A New Human Breast Carcinoma Cell Line (PMC42) with Stem Cell Characteristics. I. Morphologic Characterization. *J. Natl. Cancer Inst.,* 70, 649-661.

WHITEHEAD, R. H., MONAGHAN, P., WEBBER, L. M., BERTONCELLO, I. & VITALI, A. A. (1983b) A New Human Breast Carcinoma Cell Line (PMC42) with Stem Cell Characteristics. II. Characterization of Cells Growing as Organoids. *J. Natl. Cancer Inst.,* 71, 1193-1203.

WHITEHEAD, R. H., QUIRK, S. J., VITALI, A. A., FUNDER, J. W., SUTHERLAND, R. L., & MURPHY L. C. (1984) A New Human Breast Carcinoma Cell Line (PMC42) with Stem Cell Characteristics. III. Hormone Receptor Status and Responsiveness. *J. Natl. Cancer Inst.,* 73, 643-648.

WIJMENGA, C.& KLOMP, L.W. (2004) Molecular Regulation of Copper Excretion in the Liver. *Proc Nutr Soc*. 63, 31–39.

WOO, G. H., TAKAHASHI, M., INOUE, K., FUJIMOTO, H., IGARASHI, K., KANNO, J., HIROSE, M., NISHIKAWA, A. & SHIBUTANI, M. (2009) Cellular Distributions of Molecules with Altered Expression Specific to Thyroid Proliferative Lesions Developing in a Rat Thyroid Carcinogenesis Model. *Cancer Sci.,* 100, 617-625.

WOOTEN, L., SHULZE, R., LANCEY, R., LIETZOW, M. & LINDER, M. (1996) Ceruloplasmin is Found in Milk and Amniotic Fluid and May Have a Nutritional Role. *J. Nutr. Biochem.,* 7, 632-639.

WU, X., SINANI, D., KIM, H. & LEE, J. (2009) Copper Transport Activity of Yeast Ctr1 is Down-Regulated via its C Terminus in Response to Excess Copper. *J. Biol. Chem.,* 284, 4112-4122.

XIAO, Z., LOUGHLIN, F., GEORGE, G. N., HOWLETT, G. J. & WEDD, A. G. (2004) C-Terminal Domain of the Membrane Copper Transporter Ctr1 from Saccharomyces Cerevisiae Binds Four Cu(I) Ions as a Cuprous-Thiolate Polynuclear Cluster: Sub-Femtomolar Cu(I) Affinity of Three Proteins Involved in Copper Trafficking. *J. Am. Chem. Soc.,* 126, 3081-3090.

XIAO, Z. & WEDD, A. G. (2002) A C-Terminal Domain of the Membrane Copper Pump Ctr1 Exchanges Copper(I) with the Copper Chaperone Atx1. *Chem. Commun.,* 21, 588-589.

XU, X., PIN, S., GATHINJI, M., FUCHS, R. & HARRIS, Z. L. (2004) Aceruloplasminemia: an Inherited Neurodegenerative Disease with Impairment of Iron Homeostasis. . *Ann. N. Y. Acad. Sci.,* 1012, 299-305.

YANAGIMOTO, C., HARADA, M., KUMEMURA, H., ABE, M., KOGA, H., SAKATA, M., KAWAGUCHI, T., TERADA, K., HANADA, S., TANIGUCHI, E., NINOMIYA, H., UENO, T., SUGIYAMA, T. & SATA, M. (2011) Copper Incorporation into Ceruloplasmin is Regulated by Niemann–Pick C1 Protein. *Hepatol. Res.,* 41, 484-491.

YANG, F., FRIEDRICHS, W. E., CUPPLES, R. L., BONIFACIO, M. J., SANFORD, J. A., HORTON, W. A. & BOWMAN, B. H. (1990) Human Ceruloplasmin. Tissue-Specific Expression of Transcripts Produced by Alternative Splicing. *J. Biol. Chem.,* 265, 10780-10785.

YANG, J., QIAN, J., WEZEMAN, M., WANG, S., LIN, P., WANG, M., YACCOBY, S., KWAK, L. W., BARLOGIE, B. & YI, Q. (2006) Targeting β2-microglobulin for Induction of Tumor Apoptosis in Human Hematological Malignancies. *Cancer Cell,* 10, 295-307.

YANG, X. L., MIURA, N., KAWARADA, Y., TERADA, K., PETRUKHIN, K., GILLIAM, T. & SUGIYAMA, T. (1997) Two Forms of Wilson Disease Protein Produced by Alternative Splicing are Localized in Distinct Cellular Compartments. *Biochem. J.,* 326, 897-902.

YOUN, P., KIM, S., AHN, J. H., KIM, Y., PARK, J. D. & RYU, D. Y. (2009) Regulation of Iron Metabolism-Related Genes in Diethylnitrosamine-Induced Mouse Liver Tumors. *Toxicol. Lett.,* 184, 151-158.

ZAITSEVA, I., ZAITSEV, V., CARD, G., MOSHKOV, K., BAX, B., RALPH, A. & LINDLEY, P. (1996) The X-ray Structure of Human Serum Ceruloplasmin at 3.1 Å: Nature of the Copper Centres. *JBIC,* 1, 15-23.

ZAKHAROVA, E. T., SHAVLOVSKI, M. M., BASS, M. G., GRIDASOVA, A. A., PULINA, M. O., FILIPPIS, V. D., BELTRAMINI, M., MURO, P. D., SALVATO, B., FONTANA, A., VASILYEV, V. B. & GAITSKHOKI, V. S. (2000) Interaction of Lactoferrin with Ceruloplasmin. *Arch. Biochem. Biophys.,* 374, 222-228.

ZHOU, B. & GITSCHIER, J. (1997) hCTR1: A Human Gene for Copper Uptake Identified by Complementation in Yeast. *Proc. Natl. Acad. Sci. U. S. A. ,* 94, 7481-7486.

ZIBZIBADZE, M., BOCHORISHVILI, I., RAMISHVILI, L., MANAGADZE, L. & KOTRIKADZE, N. (2009) Investigation of Pro- and Antioxidative Systems' Changes in Blood of Patients with Prostate Tumours. *Georgian Med. News,* 169, 26-9.

ZIMNICKA, A. M., MARYON, E. B. & KAPLAN, J. H. (2007) Human Copper Transporter hCTR1 Mediates Basolateral Uptake of Copper into Enterocytes. *J. Biol. Chem.,* 282, 26471-26480.