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

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Key words copper, CTR1, lactation, human mammary gland, X-ray fluorescence microscopy, metallothionein.

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. PMC42-LA cell cultures representative of resting, lactating and suckled mammary epithelia were used to investigate the regulation of the copper uptake protein, CTR1. Both the degree of mammary epithelial differentiation (functionality) and extracellular copper concentration greatly impacted upon CTR1 expression and its plasma membrane association. In all three models (resting, lactating and suckling) there was an inverse correlation between extracellular copper concentration and the level of CTR1. Cell surface biotinylation studies demonstrated that as extracellular copper concentration increased membrane associated CTR1 was reduced. There was a significant increase in CTR1 expression (total and membrane associated) in the suckled gland model in comparison to the resting gland model, across all copper concentrations investigated (0-50 μ M). Regulation of CTR1 expression was entirely post-translational, as Quantitative Real Time PCR analyses showed no change to *CTR1* mRNA between all models and culture conditions. X-ray fluorescence microscopy on the differentiated PMC42-LA models revealed that organoid structures distinctively accumulated copper. Furthermore, as PMC42-LA cell cultures became progressively more specialised, successively more copper accumulated in organoids (resting<lactating<suckling), indicating a link between function and copper requirement. Based on previous data showing a function for CTR1 in copper uptake, we have concluded that under the influence of hormones and increased extracellular copper levels, CTR1 participates in uptake of copper by mammary epithelial cells, as a prerequisite for secretion of copper into milk.

1. Introduction

Copper in breast milk is critical for the postnatal growth and development of mammalian infants. The importance of copper at this stage is clearly illustrated by the toxic milk (*tx*) mouse model, where a defective mAtp7b (P_{1B}-type ATPase) protein impedes supply of copper into the dam's milk causing the suckling pups to become copper deficient; leading to their death after approximately two weeks of age [1, 2]. During lactation copper levels fluctuate greatly in the mother's blood independent of dietary copper uptake [3]. Concurrently, the rate of copper absorbed by the mammary gland can increase up to 20-fold during lactation [4]. Mammalian milk copper concentrations are highest the first week after birth (humans; 0.4–0.6 mg/L, rodents; 3.2–6.4 mg/L) then decrease substantially (~50%) as lactation progresses (humans; 0.2–0.3 mg/L, rodents; 0.9–2.1 mg/L) [5–7], with copper secretion into milk from the mammary gland stimulated by suckling [8]. Copper secretion into milk is regulated to not expose the suckling infant to excess or deficient levels, and thus allowing for optimal growth and development.

Until pregnancy, mammary epithelial cells remain dormant. Hormonal changes in pregnancy play an important role in modification of the mammary gland, initiating the growth and proliferation of both ducts and alveoli [9]. Estrogen and progesterone regulate the differentiation and development of mammary epithelial cells into secretory alveoli. Postpartum, estrogen and progesterone levels then reduce to pre-pregnancy levels, initiating the secretion of milk [10, 11]. Other hormones including dexamethasone, insulin and prolactin are crucial for the differentiation of mammary glands and in the induction of lactation [10, 12]. Interestingly, circulating prolactin concentrations in maternal blood positively correlate with mammary gland copper transport and prolactin has been shown to modulate the three main copper-transport proteins, mCtr1 (copper transporter 1), mAtp7a (P_{1B}-type ATPase) and mAtp7b in mouse mammary epithelial cells [8]. However, the molecular mechanisms that regulate mammary gland copper uptake and secretion into milk, including interplay between the copper-transport proteins, are not well understood. In the mammary gland, the high affinity copper uptake protein, CTR1, localises along the basolateral membrane, positioned to mediate copper uptake from maternal blood directly into mammary epithelial cells [8, 13, 14]. CTR1 is a 190-amino-acid glycosylated protein that is approximately 35kDa and has three transmembrane domains with an exofacial N-terminus and a cytosolic C-terminus [15]. Electron crystallography revealed that three CTR1 proteins can bind together to form a radially-symmetrical conical-shaped channel that is widest on the

cytosolic side [16]. The exofacial N-termini coordinate Cu(II) from the extracellular environment. Subsequently, Cu(II) is reduced to Cu(I) and passively transported through the channel to the C-termini, where it becomes available to copper chaperones like ATOX1 [17, 18]. In some mammalian cells (e.g. HEK293) elevated extracellular copper (1–100 μ M) stimulates rapid internalisation of CTR1 from the plasma membrane [19], while in other cells (e.g. HeLa and Caco-2) CTR1 localisation remains unreceptive to copper [20]. Internalisation of CTR1 could provide a quick and reversible mechanism for the regulation of cellular copper uptake.

In this study we investigated the regulation of CTR1 in cellular models of the resting, lactating and suckling mammary gland. A disadvantage of many mammary epithelial cell lines utilised for *in vitro studies* is the lack of capacity to differentiate. Previous studies demonstrated that mammary epithelial PMC42-LA cells grown on plastic retain breast-specific features including lipid granules, swollen endoplasmic reticulum and large secretory vacuoles [21, 22]. Additionally, when grown on specialised extracellular matrixes (e.g. derived from Engelbreth Holm-Swarm mouse sarcoma) PMC42-LA cells can form organoid structures similar to alveoli of the mammary gland, where secretory cells are arranged around a central lumen [23]. 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 [23]. Furthermore, differentiated PMC42-LA cells also express proteins found in milk and upon stimulation with lactational hormones express β -casein; a marker often used to identify fully differentiated lactating mammary epithelial cells [23-25]. Using PMC42-LA-based models representing resting, lactating and suckling mammary glands, we have demonstrated that both the degree of mammary epithelial differentiation and the level of extracellular copper greatly impact upon CTR1 expression and plasma membrane association. Furthermore, copper resides predominantly in organoid structures with the level rising as PMC42-LA cells become progressively more specialised towards suckled mammary gland. How CTR1 may aid the movement of copper from maternal blood into milk to sustain the rapid growth of the neonate is discussed.

2. Materials and methods

2.1. Cell culture

Breast adenocarcinoma PMC42-LA cells, a variant of the PMC42 line, were originally derived from a pleural effusion [21] and were cultured at 37°C (5% CO₂) in RPMI 1640 medium (Thermo Fisher Scientific; Melbourne, Australia) supplemented with 10% FBS (Bovogen; Melbourne, Australia). PMC42-LA cells were differentiated on porous Transwell filters (BD Falcon; Sydney, Australia) coated thinly with undiluted extracellular matrix gel from Engelbreth Holm-Swarm mouse sarcoma (EHS matrix) (Sigma-Aldrich; Sydney, Australia). Cells were treated with lactogenic hormones to obtain a model of lactating epithelia as previously described [23-25]. Briefly, cells were grown for seven days on EHS matrix and then treated with 2.7 ng/ml β -estradiol (Sigma-Aldrich) and 157 ng/ml progesterone (Sigma-Aldrich) for three days. This was followed by another three-day treatment with 1 μ g/ml dexamethasone (Sigma-Aldrich) and 0.6 μ g/ml insulin (Sigma-Aldrich), in combination with either 200 ng/ml prolactin (Jomar Diagnostic; Melbourne, Australia) to simulate lactation, or with 800 ng/ml prolactin to simulate suckling. No hormones were added to the model that recapitulates the resting breast.

2.2. Immunocytochemistry

PMC42-LA cells were grown on either coverslips (undifferentiated), or on porous Transwell cell culture inserts coated with EHS matrix (differentiated). Cells were treated as outlined in Results, then prepared for immunocytochemistry as previously described [25]. Briefly, cells grown on coverslips were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) for 10 min, permeabilised with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) for 10 min and blocked with 1% (w/v) BSA (Sigma-Aldrich) for 10 min [all in PBS (Amresco; Ohio, USA)]. Cells grown on Transwell inserts with EHS matrix were fixed in 4% (w/v) paraformaldehyde for 10min, permeabilised with 3% (v/v) Triton X-100 for 10min and blocked with 3% (w/v) BSA for 1 h. The CTR1 antibody was raised in rabbits against the first 98 amino acids of the human CTR1 protein [14, 26, 27]. Rabbit anti-CTR1 was diluted (1/1000) in 1% BSA before being applied to cells overnight at 4°C. Chicken anti-rabbit AlexaFluor 488 (Molecular Probes; Melbourne, Australia) diluted (1/5000) in 1% BSA was used for detection of primary antibodies. Cells were also counterstained with ethidium bromide (1 μ g/ml) for visualisation of nuclei. Images were collected using a Leica confocal microscope system TCS SP2 (Leica; Melbourne, Australia).

2.3. Western blot analysis

PMC42-LA cell lysates were prepared using 1% (w/v) SDS (2% for cells on Transwell inserts) in 10 mM Tris-HCl (pH 7.5) with 1x EDTA-free protease inhibitor cocktail (Roche Diagnostics; Melbourne, Australia). Lysates were homogenised by repeated passing 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. Total protein content of cell lysates were measured using the BCA Protein Assay Kit (Pierce; Melbourne, Australia) calibrated against BSA standards. Protein samples (60 µg) were fractionated using the Mini-PROTEAN® Tetra System (12% gels) (BioRad, Gladesville, Australia) and transferred to nitrocellulose (Whatman, Dassel, Germany) using the XCellIII™ blot module and the Xcell Surelock™ mini-cell system (Invitrogen; Melbourne, Australia) according to the manufacturer's instructions. The nitrocellulose membrane was then blocked using 5% (w/v) milk powder in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.6) (1 hour at room temperature) before being incubated with primary antibody diluted in TBS-T (TBS with 0.1% (v/v) Tween 20) (overnight at 4°C). The following primary antibody dilutions were used: rabbit anti-CTR1 (diluted 1/3000); mouse anti-metallothionein (diluted 1/50); (Dako; Melbourne, Australia; #M0639); mouse anti-Na/K ATPase (diluted 1/1000); (Thermo Fisher Scientific; #sc-48345); mouse anti-β-Actin (diluted 1/5000); (Sigma-Aldrich; #A5441). The following secondary antibody dilutions were used: HRP-conjugated goat anti-rabbit IgG (diluted 1/4000); (Millipore; Melbourne, Australia); HRP-conjugated goat anti-mouse IgG (diluted 1/4000); (Millipore). Proteins were detected by Immobilon enhanced chemiluminescence Substrate (Millipore) and imaged using a LAS-3000 FujiFilm Lumino-Image Analyser (Fuji Photo Film; Tokyo, Japan). Pixel intensities (arbitrary units) were quantified using Multi Gauge software [28] and levels were normalised against loading controls, being either β-actin or Na/K ATPase. At least three independent experiments were used for all comparisons.

2.4. Quantitative real time PCR (qRT-PCR)

PMC42-LA cells were harvested using trypsin solution (0.025% trypsin, 0.02% EDTA) for 5 min (incubated at 37°C). Alternatively, PMC42-LA cells grown on Transwell inserts with EHS matrix were harvested with 0.5% (w/v) dispase (Gibco; Melbourne, Australia) dissolved in PBS. Cells were incubated in dispase solution for 45 min until

detached from the EHS matrix. Harvested cells were washed three-times in PBS and RNA was then isolated using the RNeasy Mini Kit (QIAGEN; Melbourne, Australia) following the manufacturer's instructions. The RNA preparation was further purified using the DNA-free kit (Ambion; Melbourne, Australia) and the concentration determined using a Nanodrop 100 spectrophotometer (Thermo Fisher Scientific). The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Melbourne, Australia) was used to generate cDNA from the RNA preparation, following the manufacturer's instructions. Quantitative Real Time PCR was performed using a 7500 Real Time PCR system (Applied Biosystems) with 20 μ l sample volumes containing the following: 8 μ l SYBR green mastermix (Applied Biosystems), 0.3 μ M of both forward (5'-ACTCCAACAGTACCATGCAACCT) and reverse (5'-AGTTCCACATTCTTAAAGCCAAAGTAG) CTR1 primers and 20 ng of cDNA. Reactions were run on a 7500 Real Time PCR system (Applied Biosystems) on the following program: one cycle of 50°C for 2 min, one cycle of 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Relative mRNA expression was determined by comparing target CTR1 gene amplification with a β -actin housekeeping control (primers; 5'-GACAGGATGCAGAAGGAGATTACT and 5'-TGATCCACATCTGCTGGAAGGT) using the equation $2^{-\Delta\Delta CT \pm SD}$, where $\Delta\Delta CT$ was the difference between the control ΔCT and the treatment ΔCT [29].

2.5. Surface biotinylation

PMC42-LA cells grown in 6-well plates (undifferentiated), or on Transwell inserts coated with EHS matrix (differentiated), were biotinylated as previously described [19]. Briefly, cells were cooled to 4°C and washed three times with PBS supplemented with 0.1 mM CaCl₂ and 1.0 mM MgCl₂. Cells were incubated in 0.77 mg/mL EZ-Link Sulfo-NHS-SS-biotin (Pierce) in a biotinylation buffer (10 mM triethanolamine (Sigma-Aldrich), 2 mM CaCl₂, 150 mM NaCl, pH 7.5) for 25 min on ice. The cells were washed twice with a quenching buffer (PBS with 0.1 mM CaCl₂, 1.0 mM MgCl₂, 100 mM Glycine) for 20 min on ice to remove excess biotin. The cells were then lysed using a Triton X-100 containing buffer (1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5) and centrifuged at 10,000 g for 10 min. The supernatant was incubated with Streptavidin-agarose resin (0.15 volume) (Pierce) at 4°C overnight with constant agitation. The resin was washed 3 times with the lysis buffer, twice with salt (0.1% Triton X-100, 500 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5) and once with no salt (10 mM Tris, pH 7.5). Protein was then

eluted using the SDS-PAGE lysis buffer (1% SDS, 300 mM DTT, 10mM Tris-HCl, pH 7.5). Samples were analysed by Western blot as described above.

2.6. X-ray fluorescence microscopy

PMC42-LA cells were grown on ethanol sterilised 4 mm silicon nitride windows (Melbourne Centre for Nanofabrication; Melbourne, Australia), or on Transwell filters coated with EHS matrix as described above. 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 before being fixed in 4% paraformaldehyde for 10 min. Cells were then rinsed twice in PBS and again washed three times in ultrapure water (Sigma Aldrich), then air dried. Transwell filters with differentiated organoids were cut to an appropriate size and stuck to the silicon nitride windows with adhesive 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 [30, 31] on the X-ray Fluorescence Microscopy beamline [32]. This experiment used an incident X-ray beam of 14.5 keV to excite X-ray fluorescence emissions from elements of interest, in particular the first row of transition metals. Samples were scanned 10 mm from the detector, with a dwell of 62.5 ms for each 2x2 μm pixel. Scans typically covered areas of 200x200 μm . GeoPIXE version 6.3r (Library 27w32) software (CSIRO; Melbourne, Australia) was used to deconvolve the spectra, to visualise the elemental maps and to determine the localisation and levels of copper through traverse sections of the organoids. Quantitation of elemental concentrations including copper and iron was achieved by calibrating against standard reference foils of Pt and Mn.

2.7. Statistical analysis

All experiments were repeated in triplicate, and values expressed as means $\pm$ 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.

3. Results

3.1. Copper modulates *CTR1* expression and plasma membrane association in undifferentiated PMC42-LA cells.

CTR1 expression and subcellular localisation was initially investigated in undifferentiated PMC42-LA cells grown in media supplemented with various concentrations (0-50 μM) of copper (CuCl_2) for 3 days (Fig. 1). A concentration of 5 μM copper was used to match the normal level of exchangeable copper in women's plasma (between 2.7-7.7 μM) [33, 34], while 50 μM copper was added to analyse the effect of high copper. Additionally, we cultured undifferentiated PMC42-LA cells with medium supplemented with the copper-specific chelators, Bathocuproinedisulphonic acid (BCS) and D-penicillamine (DPen), to decrease available milieu copper. Note that standard growth medium contains $< 1\mu\text{M}$ copper (not shown). Quantitative Real Time PCR analyses determined that there was no significant difference ($p>0.05$) in *CTR1* mRNA levels in PMC42-LA cells grown in either low (BCS/DPen), basal (5 μM CuCl_2) or high (50 μM CuCl_2) concentrations of copper (Fig. 1A). However, there was a marked difference in the level of the monomeric CTR1 protein (35 kDa) between the copper concentrations (Fig. 1B (i)). Densitometry confirmed that there was a significant inverse correlation between extracellular copper concentration and CTR1 level (Fig. 1B (ii)). There was a ~3.5-fold increase in CTR1 in PMC42-LA cells grown in low (BCS/DPen) copper in comparison to those grown in high (50 μM CuCl_2) copper. Metallothionein (MT), a marker for elevated intracellular copper, was conversely upregulated in PMC42-LA cells grown in high (50 μM CuCl_2) copper. Metallothioneins' sequester excess intracellular copper to prevent toxicity [35].

We next employed cell surface biotinylation to determine the amount of CTR1 located at the plasma membrane of PMC42-LA cells when cultured under the same low (BCS/DPen), basal (5 μM CuCl_2) and high (50 μM CuCl_2) copper conditions (Fig. 1C (i & ii)). Biotinylated-CTR1 was precipitated from cells cultured in all treatments and again there existed a significant inverse correlation between extracellular copper concentration and the level of CTR1 (Fig. 1C (i & ii)). The copper-transport protein ATP7B served as a control and demonstrated that biotin-precipitation isolated only proteins exposed at the surface of the cell (Fig. 1C (i)). ATP7B was detected in whole cell lysate but not via biotin-precipitation, consistent with its intracellular vesicular localisation in PMC42-LA and other cell types [25, 36].

PMC42-LA cells cultured under low (BCS/DPen), basal (5 μM CuCl_2) and high (50 μM CuCl_2) copper conditions were then analysed by confocal microscopy to investigate the effect of copper on CTR1 subcellular localisation (Fig. 1D). CTR1 had a granular/punctate distribution throughout the cells in all treatment conditions. However, there was no observable CTR1 staining outlining the plasma membrane despite the fact that cell surface biotinylation experiments showed a marked increase in CTR1 being biotinylated (Fig. 1C (i & ii)). This discrepancy suggests that CTR1 may reside at the plasma membrane in an atypical punctate pattern, or transiently associate with the plasma membrane to allow biotinylation to occur but predominantly reside intracellularly. Taken together, these results demonstrate that the level of extracellular copper modulates both CTR1 expression and plasma membrane association in undifferentiated PMC42-LA cells.

3.2. Intracellular copper increases upon exposure to high copper in undifferentiated PMC42-LA cells.

X-ray fluorescent microscopy (XFM) was used to determine the intracellular distribution of copper in undifferentiated PMC42-LA cells grown in low (BCS/DPen), basal (5 μM CuCl_2) and high (50 μM CuCl_2) concentrations of media copper for 3 days (Fig. 2). Intracellular copper was undetectable (below limits) in cells grown in low (BCS/DPen) copper (Fig. 2A (i)), while a distinct and clustered signal was observed in cells grown in basal (5 μM CuCl_2) concentrations (Fig. 2A (ii)). The detection limit for copper using X-ray fluorescent microscopy was 0.03 ppm using our parameters. Intracellular copper in PMC42-LA cells cultured in high (50 μM CuCl_2) copper concentrations was clearly visible and localised to the perinuclear region rather than the cell periphery; indicative of the TGN localisation (Fig. 2A (iii)). Additionally, zinc was co-detected in all samples to better outline the position of each cell (Fig. 2A (iv-vi)). This was particularly important for cells cultured in low (BCS/DPen) and to a lesser extent basal (5 μM CuCl_2) copper, as cell structures were often indiscernible in the copper analysis alone. Image analysis using GeoPIXE software revealed that PMC42-LA cells cultured in high (50 μM CuCl_2) copper had an average copper content of $27.8 \pm 4.3\text{ppm}$ (Fig. 2B). This was 7-fold higher than in PMC42-LA cells grown in basal (5 μM CuCl_2) copper, which had approximately $4.1 \pm 1.0\text{ppm}$ (Fig. 2B).

3.3. Copper modulates CTR1 expression and plasma membrane association in differentiated PMC42-LA cells.

The PMC42-LA organoid-based models of resting and lactating mammary glands have been previously well characterised [23-25]. In this study, these models were used to determine whether the degree of mammary gland differentiation, and therefore functionality, influences Ctr1 expression and/or subcellular localisation (Fig. 3). To additionally investigate the suckled mammary gland, PMC42-LA cell were also differentiated in the presence of a high concentration of prolactin (800 ng/ml). In maternal blood, elevated levels of prolactin occur in response to suckling (mechanical stimuli) and can be sustained if feeding is frequent [10]. The three models (resting, lactating and suckling) were cultured under low (BCS/DPen), basal (5 μ M CuCl₂) and high (50 μ M CuCl₂) copper conditions for 3 days (Fig. 3). Quantitative Real Time PCR analyses determined that there was no significant difference ($p>0.05$) in *CTR1* mRNA levels between the resting, lactating and suckling models irrespective of media copper concentration (Fig. 3A). However, there were apparent post-translational changes to CTR1 between the models and in response to changes in extracellular copper (Fig. 3B (i & ii)). Firstly, in all three models (resting, lactating and suckling) there was an inverse correlation between extracellular copper concentration and the level of CTR1 (Fig. 3B (i & ii)). This relationship is analogous to what was observed in undifferentiated PMC42-LA cells (Fig. 1B (i & ii)). Secondly, there was a significant ($p<0.05$) increase in the level of CTR1 in the suckled gland model in comparison to the resting gland model, across all copper concentrations (Fig. 3B (i & ii)). The marker for elevated intracellular copper, MT, was clearly upregulated in all three models (resting, lactating and suckling) grown in high (50 μ M CuCl₂) copper. There was successively more MT expression in both lactating and suckled models ($p>0.05$), suggesting the presence of more intracellular copper as organoid functionality advances (Fig. 3C (i & ii)).

Cell surface biotinylation was used to assess the level of CTR1 associated with the plasma membrane in the three models (resting, lactating and suckling) when cultured under the same low (BCS/DPen), basal (5 μ M CuCl₂) and high (50 μ M CuCl₂) copper conditions (Fig. 3D (i & ii)). In all three models, the level of biotinylated-CTR1 significantly increased when cultured in low (BCS/DPen) media copper. There also existed a significant ($p<0.05$) increase in biotinylated-CTR1 in suckled gland in comparison to resting gland across all copper concentrations (Fig. 3D (i & ii)).

Confocal microscopy was used to visualise CTR1 localisation within the organoid structures of the three models (resting, lactating and suckling) (Fig. 4). There was no quantifiable difference in CTR1 localisation between the models or in response to being

cultured under low (BCS/DPen), basal (5 μM CuCl_2) or high (50 μM CuCl_2) copper conditions (Fig. 4A-I). CTR1 had a granular/punctate distribution throughout the cells in all treatments and it was indistinguishable whether this localisation was cytosolic, membrane bound, or a combination of both. Y-Z and X-Z section analyses (Fig. 4A-I (ii & iii)) indicated that CTR1 was predominantly basolateral. Together, these results demonstrate that both the degree of mammary epithelial differentiation and the level of extracellular copper greatly impact upon CTR1 expression and plasma membrane association.

3.4. Copper distributes to organoids in differentiated PMC42-LA models

XFM on the three PMC42-LA models (resting, lactating and suckling) revealed that organoid structures accumulate more copper than the surrounding monolayer of cells (Fig. 5). This pattern of copper distribution was particularly evident in lactating and suckled models cultured under basal (5 μM CuCl_2) and high (50 μM CuCl_2) copper conditions (Fig. 5E,F H and I). Note that regions of white, yellow and red signify high levels of copper and that Compton scattering images were used to distinguish between cells in the monolayer and those in spherical organoids (Fig. 5A-I). Compton scatter is detected when X-rays interact with lighter elements found in organic molecules like carbon, hydrogen and oxygen, with brighter regions (white, yellow, red) representing multiple layers of cells found in the organoids. As found with undifferentiated PMC42-LA cells (Fig. 2), intracellular copper was undetectable in cells grown in low (BCS/DPen) copper (below detection limit 0.03 ppm) (Fig. 5A, D and G). To substantiate that copper amassed in organoids rather than in the monolayer, traverse sections (defined by green boxes) across each XFM image covering both monolayers and organoids (Fig. 5A-I) were further analysed for copper content using GeoPIXE software (Fig. 5J-R). In all cases, the highest peak of copper content correlated with where organoids were located within the XFM image. In the same traverse sections we further examined the distribution of zinc (Fig. 5J-R). Zinc however, showed negligible variation between the XFM image areas corresponding to cell monolayers or organoids. Iron showed a similar indiscriminate pattern of distribution (not shown). Further image analysis using GeoPIXE software quantified the level of copper (Fig. 6). In all three models (resting, lactating and suckling) there was a positive correlation between extracellular copper concentration and the level of organoid copper. Again copper was undetectable (below limits) in cells grown in low (BCS/DPen) copper. Additionally, there was successively more accumulated copper in organoids in both lactating and suckled models across all copper

concentrations ($p>0.05$), suggesting that as functionality advances copper is increasingly retained.

4. Discussion

As CTR1 is postulated to mediate copper absorption from the maternal bloodstream into mammary epithelium for incorporation into milk [8], we therefore investigated CTR1 protein regulation in response to hormonal treatments simulating the different lactational states. We utilised previously developed culture models of resting and lactating mammary gland [23-25], in combination with a novel model representing the suckled state, to gain insights into CTR1 regulation and mammary epithelial copper accumulation.

X-ray fluorescence microscopy (XFM) revealed that organoid structures formed by PMC42-LA cells cultured on EHS-coated filters accumulated copper. This accumulation was exacerbated in both the lactating and suckled models and dose-dependent when all models were cultured in basal (5 μM CuCl_2) or high (50 μM CuCl_2) copper-containing media. There was up to 25-fold more copper within the centres of the organoids relative to the surrounding monolayer cells. Importantly, zinc and iron concentrations were comparable between organoids and surrounding monolayer cells, indicating a mechanism specific to copper uptake and accumulation by the mammary cells in our models. Western blot analyses of metallothionein (MT), which upregulates in response to intracellular rises in copper [35], provided further confirmation that copper concentrations increased in all three models (resting, lactating and suckling) when cultured in high (50 μM CuCl_2) copper. Taken together, these data are consistent with physiological observations described in rodents during lactation, where copper preferentially is directed towards the mammary gland for secretion into milk [4]. Our organoid data also follows the physiological observations that milk copper concentrations are altered in conjunction with circulating hormone levels during lactation. When prolactin levels are elevated during early lactation copper concentrations are high in milk and once lactation progresses circulating prolactin levels reduce as do milk copper concentrations [8].

Using the differentiated culture models we have shown that lactational hormones increased total and plasma membrane bound CTR1 protein levels in the presence of high (50 μM) copper, compared to levels of CTR1 detected in the resting model. The increase in CTR1 protein levels caused by lactational hormones correlated with the increase in intracellular copper levels. The levels of CTR1 protein increased further in the suckled model

compared to the lactating model, suggesting that prolactin can regulate CTR1. In rodents, suckling and hyperprolactinemia induce CTR1 movement to the basolateral membrane, which coincides with copper transport into the milk [8]. This, along with the data presented in this manuscript, indicates that an elevation in prolactin causes an increase in copper transport through the mammary epithelial cells. Previously, it was shown that the monomeric CTR1 can be responsive to hormones, with CTR1 levels declining in Jeg-3 placental cells upon exposure to progesterone, estrogen and insulin [26]. This response appears to contradict our PMC42-LA cell culture data. However, CTR1 in placenta is present on the foetal side rather than the maternal side and its role therefore is likely to be opposite to that in mammary epithelium; meaning that it returns excessive copper from the foetus to the maternal bloodstream [26].

It is interesting to note that HC11 mammary epithelial cells cultured in a high prolactin concentration (1 $\mu\text{g/ml}$) had no change in CTR1 protein level [8], which was probably a result of the undifferentiated state of the cells. Other copper transporting proteins, like ATP7A, can also be unresponsive to hormones in undifferentiated cells [37], while being influenced by them in differentiated cells or even tissues [38]. It is possible that the increase in CTR1 induced by exposure to hormones increases the flux of copper through the mammary cell into the milk. This may be in conjunction with ATP7B, which was reported to be a key protein involved in the delivery of intracellular copper into milk [1, 2]. ATP7B undertakes hormone and copper-induced trafficking towards the apical membrane abutting the lumen of the organoids, in the same PMC42-LA mammary culture models [25]. Following this pathway, the copper taken up by CTR1 can to be moved to the apical membrane, and secreted to milk by mechanism which yet to be determine.

Quantitative Real Time PCR analyses showed no *CTR1* mRNA changes in response to hormones, or to variations in extracellular copper levels. There have been few reports on *CTR1* mRNA levels in cultured cells, however experiments on differentiated intestinal Caco2 cells also showed no response to fluctuating copper levels [39, 40]. In further agreement with our findings, studies in mice have shown that circulating prolactin and copper concentrations have no impact on the level of *CTR1* mRNA in mammary glands [8, 41]. Dietary copper intake was also shown to have no impact on *CTR1* mRNA levels in rat pups' small intestines [42]. On the other hand, work with yeast and a small cell lung carcinoma cell line has shown that *CTR1* mRNA expression is influenced by copper [43, 44]. If mechanisms controlling copper influx by CTR1 are cell-type specific, as suggested by Molloy and Kaplan [19], then

it is possible that some cells regulate CTR1 transcriptionally, but this is not the case for mammary epithelial cells.

Our data suggests that the reduction in total protein levels in response to copper may be a result of post-translational CTR1 degradation as its transcription levels were unaltered in any of the cell treatments. Internalisation and subsequent degradation of endogenous CTR1 protein in response to elevated copper has been previously reported [45, 46]. CTR1 is important for copper uptake and is possibly the primary copper influx protein in most human cells [47, 48]. Therefore, the down-regulation of CTR1 could provide a quick and reversible mechanism for the regulation of cellular copper uptake. The addition of hormones possibly impedes 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. In our study the observation was made that the difference between the levels of CTR1 at the membrane in the low (BCS/DPen) and basal (5 μ M) copper treated cells was far greater than the difference between the total CTR1 levels of the same treatments indicating that there may be some internalisation of CTR1 in addition to its degradation. This was found in undifferentiated cultures as well as the differentiated models.

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 [14, 26, 49] with some groups also reporting perinuclear and cytoplasmic/vesicular [20, 50] distribution, however the localisation observed between cells lines seems to vary widely [20]. 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 [20, 26, 47] and in mammary gland tissues between early and late lactation when copper and hormone levels differ greatly [8]. Several studies have reported internalisation of CTR1 from the membrane following copper treatment [19, 50, 51] by clathrin dependent macropinocytosis [45, 52], which precedes proteasomal degradation [45, 53]. CTR1 internalises from the membrane into endosomes in which it has been shown to co-localise with transferrin receptor [8, 52]. 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 also have identified CTR1 at the basolateral membrane using biotinylation [19, 54]. This is consistent with mouse tissues that have shown CTR1 to be basolaterally localised in the mammary gland [8, 13, 14] and placenta [26].

In conclusion, the utilisation of a unique differentiated mammary gland model (PMC42-LA) that mimics the human breast epithelium during lactation and suckling has indicated that hormones, specifically prolactin, influence the total and membrane bound CTR1 protein levels. This is associated with an increase in intracellular copper levels, particularly in the organoids, an effect that was enhanced by the addition of lactational hormones. We proposed that while extracellular copper concentrations regulate the levels of CTR1 through internalisation and degradation, the addition of lactational hormones, predominantly prolactin, inhibits the degradation of CTR1, thus promoting copper uptake by mammary epithelial cells for subsequent secretion into milk.

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Fig. 1. The responses of CTR1 to copper treatments in undifferentiated PMC42 cells. (A) Relative *CTR1* mRNA levels under the low (BCS/DPen), basal (5 μ M CuCl₂) or high (50 μ M CuCl₂) copper conditions. The bar graph shows mean mRNA percentages $\pm$ SD from three independent experiments normalised against β -actin obtained by qRT-PCR. (B (i)) Representative CTR1 and MT Western blots of undifferentiated PMC42-LA cell extracts (60 μ g per lane) treated with low, basal and high copper. (B (ii)) Densitometric analysis of CTR1 band intensity, expressed as a mean percentage of arbitrary units $\pm$ SD compared to the basal copper treated sample and normalised against β -actin. (C (i)) This representative Western blot of extracellularly biotinylated protein extract shows CTR1 levels in undifferentiated PMC42-LA cells in response to low, basal and high copper. Negative control blot showing of a non-membrane protein ATP7B. In the biotinylated sample ATP7B was not detected, while ATP7B was detected in the whole cell lysate. (C (ii)) This graph displays the densitometric analysis of CTR1 band intensity. Values are expressed as a mean percentage of arbitrary units $\pm$ SD compared to the basal copper treated sample, normalised against Na/K ATPase. All densitometric analyses show representative results of three independent experiments. Statistical variation is indicated by the different letters above graph bars, whereas the same letters represent no significant difference ($p < 0.05$). (D) Immunolocalisation of CTR1 (green) in PMC42-LA cells treated with low (i), basal (ii) and high copper (iii). Nuclei were stained with ethidium bromide (red). Scale bar = 10 μ m.

Fig. 2. PMC42-LA intracellular copper levels in response to copper chelation and copper treatment. (A) XFM analysis showing the localisation of copper and zinc in PMC42-LA cells treated with low (i and iv), basal (ii and v) and high copper (iii and vi). The detection limit of copper and zinc was 0.03ppm for these images. The colour scale bar shows the levels of copper and zinc (ppm) from lowest (black) to highest (white). Length scale bar = 30µm. (B) Analysis of the XFM images using GeoPIXE software revealed the levels of copper in the cells of each treatment. Statistical variation is indicated by the different letters above graph bars, whereas the same letters represent no significant difference ($p < 0.05$).

Fig. 3. The copper and hormonal responses of CTR1 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 $\pm$ SD compared to the basal copper treated cells of the resting model. *CTR1* mRNA levels were normalised against β -actin. L = low copper, B = basal copper, H = high copper. (B (i)) Representative Western blots of total protein extract (60 μ g per lane) showing the CTR1 bands in the resting breast model, lactating model and suckled model treated with low, basal and high copper. The β -actin blot demonstrates relative loading of samples. (B (ii)) The CTR1 densitometry graph bar show mean intensity of CTR1 bands expressed as a percentage of arbitrary units $\pm$ SD compared to the basal copper treatment of the resting model. Densitometric analysis of CTR1 bands were normalised against β -actin. (C (i)) Representative Western blots of total protein extract showing the MT bands in the resting breast model, lactating model and suckled model treated with low, basal and high copper. The β -actin blot demonstrates relative loading of samples. (C (ii)) The densitometry graph shows mean intensity of MT bands expressed as a percentage of arbitrary units $\pm$ SD compared to the high copper treatment of the resting model. Densitometric analysis of MT bands were normalised against β -actin. (D (i)) 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 low, basal and high copper. (D (ii)) Densitometric analysis shows mean intensity of CTR1 bands expressed as a percentage of arbitrary units $\pm$ SD compared to the basal copper treatment of the resting model. Densitometric analysis of CTR1 bands were normalised against Na/K ATPase. All blots are representative of four independent experiments. Statistical variation is indicated by the different letters above graph bars, whereas the same letters represent no significant difference ($p < 0.05$).

Fig. 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 low, basal and high copper (A, B and C). CTR1 protein localisation in the lactating breast cell model in response to low, basal copper and high copper (D, E and F). CTR1 localisation within the suckled breast cell model in response to low, basal and high 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 = 20µm.

Fig. 5. Copper distribution and levels within differentiated PMC42-LA organoids. X-ray fluorescence images show the location of copper within the low (A, D and G), basal (B, E and H) and high 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). Compton represents the X-ray scatter from lighter elements found in organic molecules, such as carbon, hydrogen and oxygen, and provides impression of the general structures of cell culture model. Compton images accompany the XFM copper images to show the position of each of the organoids. The colour scale bar shows the levels of copper (ppm) and Compton (arbitrary units $\times 10^3$) from lowest (black) to highest (white). Length scale bar = 30 μ m. The green box on each XFM image indicates the region of the traverse section displayed in figures 5J-R. Traverse sections through the organoids reveal the change in copper levels between the monolayers of cells and organoid structures found in differentiated PMC42-LA cells. For each cell model treated with low (J, M and P), basal (K, N and Q) and high 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 5A-I. The detection limit of copper and zinc was 0.03ppm for these images. The x-axis shows distance along the traverse line in micrometers and the y-axis shows the levels of each element in ppm.

Fig. 6. The copper levels of differentiated PMC42-LA cells in response to copper and lactational hormones. Analysis of the XFM images using GeoPIXE software revealed the levels of copper in the cells of each treatment. The graph displays the levels of copper within the organoids of low, basal and high copper treatment resting, lactating and suckled models. Statistical variation is indicated by different letters above graph bars ($p < 0.05$).

References

- [1] Michalczyk AA, Rieger J, Allen KJ, Mercer JFB, Ackland ML. 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* 2000;352:565-71.
- [2] Rauch H. Toxic Milk, a New Mutation Affecting Copper Metabolism in the Mouse. *J Hered* 1983;74:141-4.
- [3] Dorea JG. Iron and copper in human milk. *Nutrition* 2000;16:209-20.
- [4] Donley SA, Ilagan BJ, Rim H, Linder MC. Copper Transport to Mammary Gland and Milk During Lactation in Rats. *Am J Physiol Endocrinol Metab* 2002;283:667-75.
- [5] Friel JK, Andrews WL, Jackson SE, Longerich HP, Mercer C, McDonald A, et al. Elemental Composition of Human Milk From Mothers of Premature and Full-Term Infants During the First 3 Months of Lactation. *Biol Trace Elem Res* 1999;67:225-47.
- [6] Salmenpera L, Perheentupa J, Pakarinen P, Siimes MA. Cu Nutrition in Infants During Prolonged Exclusive Breast-Feeding: Low Intake but Rising Serum Concentrations of Cu and Ceruloplasmin. *Am J Clin Nutr* 1986;43:251-7.
- [7] Linder MC, Wooten L, Cerveza P, Cotton S, Shulze R, Lomeli N. Copper Transport. *Am J Clin Nutr* 1998;67:965S-71S.
- [8] Kelleher SL, Lonnerdal B. Mammary Gland Copper Transport is Stimulated by Prolactin Through Alterations in Ctr1 and Atp7A Localization. *Am J Physiol Regul Integr Comp Physiol* 2006;291:1181-91.
- [9] Lamote I, Meyer E, Massart-Leën AM, Burvenich C. Sex Steroids and Growth Factors in the Regulation of Mammary Gland Proliferation, Differentiation, and Involution. *Steroids* 2004;69:145-59.
- [10] Neville MC, McFadden TB, Forsyth I. Hormonal Regulation of Mammary Differentiation and Milk Secretion. *J Mammary Gland Biol Neoplasia* 2002;7:49-66.
- [11] Rillema JA. Development of the Mammary Gland and Lactation. *Trends Endocrinol Metab* 1994;5:149-54.
- [12] Hovey RC, Trott JF, Vonderhaar BK. Establishing a Framework for the Functional Mammary Gland: From Endocrinology to Morphology. *J Mammary Gland Biol Neoplasia* 2002;7:17-38.

- [13] Kelleher SL, Lonnerdal B. Marginal Maternal Zn Intake in Rats Alters Mammary Gland Cu Transporter Levels and Milk Cu Concentration and Affects Neonatal Cu Metabolism. *J Nutr* 2003;133:2141-8.
- [14] Llanos RM, Michalczyk AA, Freestone DJ, Currie S, Linder MC, Ackland ML, et al. Copper Transport During Lactation in Transgenic Mice Expressing the Human ATP7A Protein. *Biochem Biophys Res Commun* 2008;372:613-7.
- [15] Klomp AEM, Juijn JA, Gun LTMvd, Berg IETvd, Berger R, Klomp LWJ. The N-Terminus of the Human Copper Transporter 1 (hCTR1) is Localized Extracellularly, and Interacts with Itself. *Biochem J* 2003;370:881-9.
- [16] de Feo CJ, Aller SG, Siluvai GS, Blackburn NJ, Unger VM. Three-Dimensional Structure of the Human Copper Transporter hCTR1. *Proc Natl Acad Sci USA* 2009;106:4237-42.
- [17] Ferruzza S, Sambuy Y, Ciriolo MR, Martino AD, Santaroni P, Rotilio G, et al. Copper Uptake and Intracellular Distribution in the Human Intestinal Caco-2 Cell Line. *Biometals* 2000;13:179-85.
- [18] Howell SB, Safaei R, Larson CA, Sailor MJ. Copper Transporters and the Cellular Pharmacology of the Platinum-Containing Cancer Drugs. *Mol Pharmacol* 2010;77:887-94.
- [19] Molloy SA, Kaplan JH. Copper-dependent Recycling of hCTR1, the Human High Affinity Copper Transporter. *J Biol Chem* 2009;284:29704-13.
- [20] Klomp AEM, Tops BBJ, Berg IETvd, Berger R, Klomp LWJ. Biochemical Characterization and Subcellular Localization of Human Copper Transporter 1 (hCTR1). *Biochem J* 2002;364:497-505.
- [21] Whitehead RH, Bertoncello I, Webber LM, Pedersen JS. A New Human Breast Carcinoma Cell Line (PMC42) With Stem Cell Characteristics. I. Morphologic Characterization. *J Natl Cancer Inst* 1983;70:649-61.
- [22] Whitehead RH, Monaghan P, Webber LM, Bertoncello I, Vitali AA. A New Human Breast Carcinoma Cell Line (PMC42) with Stem Cell Characteristics. II. Characterization of Cells Growing as Organoids. *J Natl Cancer Inst* 1983;71:1193-203.
- [23] Ackland ML, Michalczyk A, Whitehead RH. PMC42, A Novel Model for the Differentiated Human Breast. *Exp Cell Res* 2001;263:14-22.
- [24] Ackland ML, Ward J, Ackland CM, Greaves M, Walker M. 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* 2003;39:428-33.
- [25] Michalczyk A, Bastow E, Greenough M, Camakaris J, Freestone D, Taylor P, et al. ATP7B Expression in Human Breast Epithelial Cells is Mediated by Lactational Hormones. *J Histochem Cytochem* 2008;56:389-99.
- [26] Hardman B, Manuelpillai U, Wallace EM, Monty J-F, Kramer DR, Kuo YM, et al. Expression, Localisation and Hormone Regulation of the Human Copper Transporter hCTR1 in Placenta and Choriocarcinoma Jeg-3 Cells. *Placenta* 2006;27:968-77.
- [27] Ke B-X, Llanos RM, Mercer JFB. ATP7A Transgenic and Nontransgenic Mice are Resistant to High Copper Exposure. *J Nutr* 2008;138:693-7.
- [28] Woo G-H, Takahashi M, Inoue K, Fujimoto H, Igarashi K, Kanno J, et al. Cellular Distributions of Molecules with Altered Expression Specific to Thyroid Proliferative Lesions Developing in a Rat Thyroid Carcinogenesis Model. *Cancer Sci* 2009;100:617-25.
- [29] Livak JK, Schmittgen DT. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods* 2001;25:402-8.
- [30] Kirkham R, Dunn PA, Kuczewski A, Siddons DP, Dodanwala R, Moorhead G, et al. The Maia Spectroscopy Detector System: Engineering for Integrated Pulse Capture, Low-Latency Scanning and Real-Time Processing. *AIP Conf Proc* 2010;1234:240-3
- [31] Ryan CG, Kirkham R, Hough RM, Moorhead G, Siddons DP, de Jonge MD, et al. Elemental X-Ray Imaging Using the Maia Detector Array: the Benefits and Challenges of Large Solid-Angle. *Nucl Instrum Methods Phys Res Sect A: Accelerators, Spectrometers, Detectors and Associated Equipment* 2010;619:37-43.
- [32] Paterson D, de Jonge MD, Howard DL, Lewis W, McKinlay J, Starritt A, et al. The X-Ray Fluorescence Microscopy Beamline at the Australian Synchrotron. *AIP Conf Proc* 2011;1365:219-22.
- [33] Linder MC. *Biochemistry of Copper*. New York: Plenum Press, 1991.
- [34] Linder MC. *Biochemistry and Molecular Biology of Copper in Mammals*. Totowa, NJ: Humana Press, 2002.
- [35] Coyle P, Philcoxa JC, Careya LC, Rofea AM. Metallothionein: The Multipurpose Protein. *Cell Mol Life Sci* 2002;59:627-47.
- [36] Cater MA, La Fontaine S, Shield K, Deal Y, Mercer JFB. ATP7B Mediates Vesicular Sequestration of Copper: Insight Into Biliary Copper Excretion. *Gastroenterology* 2006;130:493-506.

- [37] Ackland ML, Cornish EJ, Paytner JA, Grimes A, Michalczyk A, Mercer JFB. Expression of Menkes Disease Gene in Mammary Carcinoma Cells. *Biochem J* 1997;328:237-43.
- [38] Ackland ML, Anikijenko P, Michalczyk A, Mercer JFB. Expression of Menkes Copper-Transporting ATPase, MNK, in the Lactating Human Breast: Possible Role in Copper Transport into Milk. *J Histochem Cytochem* 1999;47:1553-61.
- [39] Bauerly KA, Kelleher SL, Lonnerdal B. Functional and Molecular Responses of Suckling Rat Pups and Human Intestinal Caco-2 Cells to Copper Treatment. *J Nutr Biochem* 2004;15:155-62.
- [40] Tennant J, Stansfield M, Yamaji S, Srai SK, Sharp P. Effects of Copper on the Expression of Metal Transporters in Human Intestinal Caco-2 Cells. *FEBS Lett* 2002;527:239-44.
- [41] Gambling L, Dunford S, McArdle HJ. Iron Deficiency in the Pregnant Rat has Differential Effects on Maternal and Fetal Copper Levels. *J Nutr Biochem* 2004;15:366-72.
- [42] Bauerly KA, Kelleher SL, Lonnerdal B. Effects of Copper Supplementation on Copper Absorption, Tissue Distribution and Copper Transporter Expression in an Infant Rat Model. *Am J Physiol Gastrointest Liver Physiol* 2005;288:1007-14.
- [43] Kirchman PA, Botta G. Copper Supplementation Increases Yeast Life Span Under Conditions Requiring Respiratory Metabolism. *Mech Ageing Dev* 2007;128:187-95.
- [44] Song I-S, Chen HHW, Aiba I, Hossain A, Liang ZD, Klomp LWJ, et al. Transcription Factor Sp1 Plays an Important Role in the Regulation of Copper Homeostasis in Mammalian Cells. *Mol Pharmacol* 2008;74:705-13.
- [45] Holzer AK, Howell SB. The Internalization and Degradation of Human Copper Transporter 1 following Cisplatin Exposure. *Cancer Res* 2006;66:10944-52.
- [46] Safaei R, Maktabi MH, Blair BG, Larson CA, Howell SB. Effects of the Loss of Atox1 on the Cellular Pharmacology of Cisplatin. *J Inorg Biochem* 2009;103:333-41.
- [47] Eisses JF, Chi Y, Kaplan JH. Stable Plasma Membrane Levels of hCTR1 Mediate Cellular Copper Uptake. *J Biol Chem* 2005;280:9635-9.
- [48] Lee J, Petris MJ, Thiele DJ. Characterization of Mouse Embryonic Cells Deficient in the Ctr1 High Affinity Copper Transporter. *J Biol Chem* 2002;277:40253-9.
- [49] Maryon EB, Molloy SA, Kaplan JH. O-Linked Glycosylation at Threonine 27 Protects the Copper Transporter hCTR1 from Proteolytic Cleavage in Mammalian Cells. *J Biol Chem* 2007;282:20376-87.

- [50] Guo Y, Smith K, Petris MJ. Cisplatin Stabilizes a Multimeric Complex of the Human Ctr1 Copper Transporter. *J Biol Chem* 2004;279:46393-9.
- [51] Guo Y, Smith K, Lee J, Thiele DJ, Petris MJ. Identification of Methionine-rich Clusters That Regulate Copperstimulated Endocytosis of the Human Ctr1 Copper Transporter. *J Biol Chem* 2004;279:17428-33.
- [52] Petris MJ, Smith K, Lee J, Thiele DJ. Copper-stimulated Endocytosis and Degradation of the Human Copper Transporter, hCtr1. *J Biol Chem* 2003;278:9639-46.
- [53] Jandial DD, Farshchi-Heydari S, Larson CA, Elliott GI, Wrasidlo WJ, Howell SB. Enhanced Delivery of Cisplatin to Intraperitoneal Ovarian Carcinomas Mediated by the Effects of Bortezomib on the Human Copper Transporter 1. *Clin Cancer Res* 2009;15:553-60.
- [54] Zimnicka AM, Maryon EB, Kaplan JH. Human Copper Transporter hCTR1 Mediates Basolateral Uptake of Copper into Enterocytes. *J Biol Chem* 2007;282:26471-80.

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