Chapter 2

NUTRIENT TRANSFER: MAMMARY GLAND REGULATION

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1. INTRODUCTION

Breast-milk is arguably the ultimate functional food providing the nursing infant with basic nutrition as well as a complex mixture of immunomodulatory components, bioactive compounds and a vast array of hormones[1]. Having been breast-fed as an infant has been associated with enhanced cognitive development[2] and may also provide protection against cardiovascular disease[3, 4], obesity[5] and type 1[6] and type 2 diabetes[7] later in life. Appropriate trace element intake is essential for optimal growth and development and as such may play a role in some of the positive outcomes associated with breastfeeding. Breast-fed infants are entirely dependent upon the mother to provide an appropriate trace element supply and evidence indicates that trace element requirements of term infants are generally met by exclusive breast-feeding through about the first 6 months of life[8]. After 6 months of age, introduction of complementary foods with adequate trace element content is essential to meet the nutritional needs of the growing infant. This is due in part to milk iron (Fe), zinc (Zn) and copper (Cu) concentrations declining throughout lactation[9]. Furthermore, milk Fe, Zn and Cu concentrations are relatively refractory to maternal trace mineral status[10], even when the maternal diet varies considerably[11]. There is currently little information regarding the mechanisms through which the mammary gland regulates milk trace element concentrations. Similarities between humans

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and rodents[12] allow us to use rodent models to examine the regulation of mammary gland mineral transport. Recently, several transporters for Fe, Zn and Cu have been found to control trace element uptake and efflux in various cell types. We have utilised the lactating rat to determine changes in mammary gland Fe, Cu and Zn transporter expression and localisation that occur throughout lactation and in response to maternal trace mineral deficiency in hopes of elucidating some of the changes which may be occurring in lactating women.

2. MAMMARY GLAND IRON TRANSPORT

Adequate Fe intake is essential for optimal growth, hematopoiesis and cognitive development during infancy. Iron deficiency anemia is the most common nutrient deficiency and is estimated to affect 1 to 2 billion people worldwide.[13] While maternal Fe deficiency has not been associated with neonatal Fe deficiency anemia *per se*, neonatal Fe stores are decreased[14] leaving the newborn at increased risk for Fe deficiency if Fe intake is inadequate. Milk Fe concentration in humans and rats normally declines throughout the course of lactation.[9,15] However, little correlation between maternal Fe status and milk Fe concentration in lactating women[11] or between marginal Fe deficiency and milk Fe concentration in rats has been observed[16], indicating that mammary gland Fe transport is a tightly regulated process thus ensuring appropriate Fe transfer to the neonate.

Cellular Fe transport is tightly regulated and consists of Fe uptake across the plasma membrane, the partitioning of Fe into specific intracellular pools and Fe export across the plasma membrane in some cell types such as the secretory mammary epithelial cell. Iron uptake into the mammary gland is facilitated by transferrin receptor (TfR). However, no correlation between milk Fe concentration and TfR expression has been observed, suggesting that the control of milk Fe level occurs following Fe uptake into the mammary gland.[17] Once diferric transferrin binds to TfR at the cell surface[17], the transferrin–TfR complex is internalised in clathrin-coated vesicles that fuse with acidic endosomes. The acidic environment facilitates the release of Fe from the transferrin–TfR complex within the endosomal vesicle. Iron is transported out of the endosome by divalent metal transporter 1 (DMT1).[18-19]

While we have determined that DMT1 is expressed in the mammary gland[16], its localisation and the role it plays in mammary gland Fe metabolism have not yet been characterised. Once Fe has entered the mammary epithelial cell it may partition into a chelatable Fe pool or participate in a multitude of cellular processes such as sequestration into

ferritin (Ft) for storage, incorporation into Fe containing proteins in the endoplasmic reticulum (ER) or export across the luminal membrane into milk. How Fe secretion into milk is facilitated is currently unknown. However, ferroportin (FPN) or IREG1 is localised to the endoplasmic reticulum in reticuloendothelial cells where it is assumed to facilitate Fe transport into an intracellular vesicle prior to secretion.[20] We have determined that FPN is expressed in the mammary gland [16] and speculate that mammary gland FPN similarly transports Fe into secretory vesicles destined for export into milk.

To address questions regarding the regulation of mammary gland Fe transport we have used the lactating rat as a model to localise DMT1 and FPN and determine changes in mammary gland Fe transporter expression throughout lactation and in response to maternal Fe deficiency. Using immunohistochemistry, we have determined that both DMT1 and FPN are localised to intracellular vesicles and this cellular localisation in combination with predicated membrane topology suggests that DMT1 may play a role in endosomal Fe export while FPN may indirectly participate in the secretion of Fe into milk.[21] As mentioned previously, milk Fe concentration declines throughout lactation and this decline is associated with declining levels in TfR and FPN expression. In contrast, mammary gland Fe concentration and DMT1 expression both remain constant throughout lactation further suggesting that DMT1 may play a role in mediating cellular Fe pools.[16] These results taken together suggest that the decline in milk Fe concentration that occurs throughout normal lactation results from decreased Fe uptake and secretion from the mammary gland into milk and not from tissue Fe depletion and may partially reflect the improvement in maternal Fe status that occurs during the postnatal period.[22] Maternal Fe deficiency in lactating rats reduced mammary gland Fe levels during lactation and similar to observations in lactating women, milk Fe concentration was not affected. The maintenance of milk Fe level was associated with decreased mammary gland ferritin and DMT1 expression while TfR and FPN expression were not affected. These results further suggest that the primary regulators of milk Fe secretion may be TfR and FPN and indicate that milk Fe levels are maintained during Fe deficiency due to an uncoupling of the “normal” tissue Fe-responsive regulatory mechanisms in the mammary gland.

3. MAMMARY GLAND COPPER TRANSPORT

Copper (Cu) plays an essential role as a cofactor for enzymes that generate cellular energy, cross-link connective tissue and mobilise cellular iron.[23] A large amount of Cu is accreted by the fetal liver [24] and is effectively

mobilised during early neonatal life[25]. However, studies in rodents indicate that total body Cu content increases during suckling suggesting that Cu must be absorbed from their diet as well[26]. During lactation, milk provides the sole source of Cu to the offspring; however, milk Cu concentration decreases as lactation progresses in both rodents[12] and humans[27]. Currently, the mechanisms in the mammary gland which facilitate the decrease in milk Cu concentration are not understood.

Three mammalian Cu-specific transport proteins have been identified in the mammary gland[28-30]. The Menkes Cu ATPase (ATP7A) belongs to the P-type ATPase family of transmembrane proteins, and mutations in the ATP7A gene are associated with impaired cellular Cu export[31]. ATP7A expression is ubiquitous and its gene product is localised to both a perinuclear and vesicular compartment in mammary glands of mice and humans in the non-lactating state[29, 32]. However, during lactation mammary gland ATP7A expression is increased and ATP7A protein re-localises to the plasma membrane[29] suggesting that mammary gland ATP7A plays an active role in mammary gland Cu transport during lactation. The Wilson Cu ATPase (ATP7B) also belongs to the P-type ATPase family and is homologous to ATP7A[30]. Individuals with Wilson disease have mutations in the ATP7B gene which eliminates the ability of ATP7B protein to appropriately localise to an intracellular compartment in the liver, resulting in impaired biliary Cu excretion and subsequent hepatotoxicity[31]. In the rat mammary gland during mid-lactation, ATP7B is localised to an intracellular compartment and to the luminal membrane of secretory mammary epithelial cells[28]. Similar to observations in patients with Wilson disease, a murine mutation in ATP7B (toxic milk, *tx*) results in defective ATP7B translocation in the mammary gland thus impairing Cu export into milk (~20 % of normal). This mis-localisation of ATP7B in the mammary gland results in neonatal death from Cu deficiency suggesting it plays a major role in mammary gland Cu export into milk. [30] Prior to export into milk, Cu must be imported into the mammary gland; however, the mechanisms the mammary gland uses to accomplish Cu import are not well understood. In the circulation, Cu is tightly complexed with ceruloplasmin (Cp), associated with albumin and, to a lesser degree, small molecular weight ligands such as amino acids[25]. Recently Ctr1, an essential Cu import protein, has been identified and found to be expressed in all tissues examined[33, 34] including the mammary gland[28]. Studies in transfected cell models indicate that Ctr1 imports Cu^{+1} with high affinity[33,35] and import is believed to require multimerisation of several Ctr1 proteins[36], possibly forming a channel[37]. Additionally, recent evidence indicates that Ctr1 is vesicular and is endocytosed and degraded in response to physiological levels of extracellular Cu[38], presumably providing a rapid method of modulating Cu

import. Similar to what has been observed in numerous cell types[37], we have determined that Ctr1 in the mammary gland is localised to both the plasma membrane and intracellular vesicles[28].

We used a lactating rat model and characterised changes in Cu transporter expression and localisation during lactation. Similar to observations in humans[9], milk and plasma Cu concentration declined through lactation as did mammary gland Cu levels. The decrease observed in milk Cu level as lactation progresses may be primarily a result of the internalisation of Ctr1 from the serosal membrane as lactation progresses in combination with reduced Cu availability from maternal circulation due to decreasing plasma Cu concentration. These changes may facilitate the depletion of mammary gland Cu levels. Furthermore, the protein level and localisation of ATP7B is maintained throughout lactation while the amount of ATP7A protein is higher during early compared to late lactation. Although the role of ATP7A in mammary gland Cu export is currently unknown, high expression of ATP7A during early lactation may facilitate enhanced Cu secretion into milk during this period, while the longitudinal decrease in ATP7A level may reduce the ability of the mammary gland to secrete Cu into milk as lactation proceeds. However, the possibility that ATP7A plays a yet unknown role in mammary gland Cu transport cannot be excluded.

While Cu deficiency is uncommon, marginal Zn intake is very common and Zn deficiency during pregnancy and lactation has been associated with secondary affects on Cu metabolism in the offspring. Research from our group has recently demonstrated an inverse correlation between maternal Zn status and milk Cu concentration[28]; however, the underlying mechanisms are unknown. We used the lactating rat as a model and determined that marginal maternal Zn intake similarly resulted in increased milk Cu concentration and Cp activity. Furthermore, Zn deficient rats had increased mammary gland Ctr1, ATP7A and ATP7B expression and also relocalised ATP7A to larger vesicles in the mammary gland, potentially increasing Cu secretion into secretory vesicles. Thus, suboptimal maternal status of one trace element may affect the milk concentration of other essential trace elements, emphasising the need for adequate maternal nutrition of multiple trace elements to ensure optimal trace element transfer to the nursing infant.

4. MAMMARY GLAND ZINC TRANSPORT

Zinc is a nutrient required for many proteins involved in DNA synthesis, protein synthesis, mitosis and cell division. Adequate Zn supply is particularly important during the periods of rapid neonatal growth and

development as illustrated by observations of early neonatal death associated with low milk Zn levels in lethal milk (*lm*) mice.[39] During lactation, a substantial amount of Zn is taken up by the human mammary gland and secreted into milk (0.5-1 mg/d), facilitating the movement of almost twice the amount of Zn that is transferred daily across the placenta to the fetus during pregnancy,[40] which illustrates the extraordinary activity of mammary gland Zn transport. Furthermore, milk Zn concentration is maintained over a wide range of dietary Zn intake,[41-42] which suggests that mammary gland Zn import and export are tightly coordinated in order to provide adequate Zn to the nursing infant. Interestingly, although plasma Zn concentration increases, milk Zn concentration decreases throughout the normal course of lactation in both rodents and humans;[12] however, the transport mechanisms that regulate this longitudinal decrease are not well understood.[43]

Recently, a number of mammalian proteins have been described which participate in Zn trafficking from the cytosol across membranes.[44] These are divided into two distinct families. The ZnT family of Zn transporters is a member of the larger cation diffusion facilitator family (CDF) and currently contains 7 members (ZnT-1 through ZnT-7). With the exception of ZnT-5[45], they are structurally similar having six transmembrane domains and a histidine-rich domain that is believed to play a key role in Zn binding; however, the specific mechanisms these transporters utilise to transport Zn remain unknown. The importance of optimal mammary gland Zn transfer is recognised by the early death from severe Zn deficiency of pups suckled from dams exhibiting a nonsense mutation in the Zn transporter ZnT-4, known as the lethal milk (*lm*) mouse.[39] Although this suggests that ZnT-4 plays an important role in facilitating milk Zn secretion, observations that milk from these mice still contains measurable amounts of Zn (~50% of normal)[46] and that pup survival can be improved by maternal Zn supplementation, indicate that the mammary gland can utilise other Zn transport mechanisms to facilitate the export of Zn into milk. Thus far, ZnT-1 is the only Zn transporter that has been implicated in cellular Zn export.[47] ZnT-1 may therefore export Zn across both the serosal and luminal membranes of the mammary epithelial cell, the cell-type responsible for the secretion of milk components during lactation.[48] ZnT-2 is expressed in the mammary gland[48] and is primarily associated with the luminal membrane, possibly exporting Zn from the cytosol into secretory vesicles.[49-50] However, the physiological significance of this vesicular Zn sequestration remains obscure.

The initial step in milk Zn secretion is Zn import from the maternal circulation into the mammary gland. Although Zip1 expression is ubiquitous, abundant expression of Zip2, Zip3 and Zip4 is tissue-specific.[51] The expression of Zip3 is restricted to tissues with an unusually high requirement

for Zn such as brain, eye, pancreas and thymus. Additionally, we have detected Zip3 expression in the mammary gland and like other Zip family members,[52-55] Zip3 is localised to the plasma membrane in mammary epithelial cells. Taken together, these data suggest that Zip3 may play a unique regulatory role in mammary gland Zn import and thus ultimately in milk Zn secretion.

We used the lactating rat as a model and determined that, similar to observations in humans,[56] the plasma Zn concentration of lactating rats increases to pre-pregnancy levels as lactation progresses. Concurrent with the increasing plasma Zn level, mammary gland Zn concentration, ZnT-1 and ZnT-2 expression increase while ZnT-4 and Zip3 expression peaks during early lactation and then declines, but remains significantly higher than during early lactation.[48] While ZnT-1 expression increases throughout lactation, the formation of two distinct ZnT-1 complexes of different size may help to explain differential cellular localisation. Interestingly, the intensity of luminal-associated ZnT-1 staining is particularly high during early lactation and declines as lactation continues. This suggests that ZnT-1 may play a significant role in mediating the transfer of Zn into milk during early lactation and that its contribution diminishes as lactation progresses. While the expression of ZnT-2 slightly increases throughout lactation and the staining intensity of ZnT-2 at the serosal membrane remains constant, the intensity of ZnT-2 staining at the luminal membrane decreases through lactation. This decline in luminal staining as lactation proceeds provides an additional mechanistic explanation for the decline in milk Zn concentration. ZnT-4 in the mammary gland is also localised to both serosal and luminal mammary cell compartments; however, its relative distribution shifts from the luminal membrane during early lactation to a more even intracellular distribution during late lactation possibly reducing its overall contribution to milk Zn secretion. The peak in ZnT-4 and Zip3 expression during early lactation also suggests that mammary gland Zn uptake and milk Zn secretion are enhanced via these transporters during early lactation and further provides a mechanistic explanation behind the decline in milk Zn levels that has been observed.

Milk Zn level is maintained over a wide range of dietary Zn intake and most studies have failed to show a positive effect of Zn supplementation on milk Zn level, despite increased plasma Zn levels. This indicates that the regulation of milk Zn secretion is tightly controlled. Some studies have observed an inverse relationship between milk Zn (which is high) and plasma Zn (which is low) in women from developing countries; however, the mechanisms the mammary gland uses to facilitate this regulation is unknown. Using the lactating rat as a model, we determined effects of low Zn intake on mammary gland Zn transporter expression at mid-lactation and

found that similar to observations in humans, although plasma Zn levels are reduced, milk Zn concentration is maintained during marginal Zn intake. Furthermore, we speculate that milk Zn level may be homeostatically maintained via decreased Zn export back across the serosal membrane into maternal circulation, as ZnT-1 expression is decreased, and increased Zn secretion into milk, as ZnT-4 expression is increased. However, this effect is dependent upon the severity of Zn deficiency as once Zn intake is further compromised, milk Zn level decreases and is associated with decreased expression of Zip3, ZnT-1, ZnT-2 and ZnT-4, suggesting a threshold to which the mammary gland can respond to adequately maintain milk Zn levels.

4.1 Regulation of Zip3 and ZnT-4 by prolactin

Within the mammary gland, the highly specialised, secretory mammary epithelial cell is responsible for the secretion of milk components and thus facilitates the transport of large amounts of Zn from the maternal circulation into milk. Differentiation of proliferating mammary epithelial cells into a fully functional, secretory cell-type is hormonally regulated and essential for preparing these cells for secretion.[57] Furthermore, once differentiated, secreting mammary epithelial cells require episodic hormonal stimulation in order to maintain the expression, production and secretion of many milk components[58] similar to the requirements for galactopoeisis[59,60]. During lactation, prolactin (PRL), primarily secreted by the anterior pituitary gland[61-63], is responsible for regulating milk protein synthesis and maintaining lactation,[64-65] and circulating PRL levels decline as lactation progresses.

As we have characterised changes in Zn transporters that occur during lactation, we aim to further understand the regulatory mechanisms which facilitate these changes. The redundancy in the mammary gland Zn transport system has led us to question the unique role each Zn transporter plays in this process. The use of gene silencing techniques has greatly aided the understanding of many complex biological processes[66] and is becoming an increasingly common tool in evaluating protein functionality and essentiality in specific cell types. Using gene silencing we reduced Zip3 expression in cultured mouse mammary epithelial cells by ~80% and subsequently decreased Zn uptake, demonstrating that Zip3 facilitates Zn import into mammary epithelial cells. Furthermore, decreased cell viability following Zip3 knock-down illustrates the biological essentiality of Zip3 in mammary epithelial cells and may reflect the unique requirement for enhanced Zn transport via Zip3 in this highly specialised cell type. As mentioned previously, PRL secretion is episodic and circulating PRL level declines

throughout lactation, and thus we speculate that PRL may play a role in mediating changes in milk Zn (as well as Fe and Cu) concentrations. To investigate the mechanisms through which PRL affects Zip3 and ZnT-4 we used cultured mouse mammary epithelial cells and observed that PRL exposure transiently stimulated both serosal Zn uptake and luminal Zn export in these cells. However, this increase in Zn transport was associated with increased ZnT-4 expression but decreased Zip3 expression indicating that increased Zn transporter protein levels was not the only explanation for the observed increase in Zn transport. Using confocal microscopy we have determined effects of PRL on Zip3 and ZnT-4 localisation in mouse mammary epithelial cells. Similar to the localisation of Zip3 in lactating rat mammary gland[48], Zip3 was localised to the plasma membrane and to a vesicular compartment of mammary epithelial cells, indicating that Zip3 may episodically facilitate mammary epithelial cell Zn import[52]. Furthermore, PRL exposure facilitates the movement of Zip3-associated vesicles towards the plasma membrane presumably increasing Zn uptake into the cell. ZnT-4 on the other hand, generally stains throughout the entire mammary epithelial cell, but stains in very tight association with a perinuclear compartment following PRL exposure.

One important question that arises is: how does PRL mediate these transcriptional, translational and post-translational effects on Zip3 and ZnT-4? PRL binds to PRL receptor and through a series of phosphorylation events can stimulate the JAK2/STAT5[67-68] and MAP kinase[69] pathways, ultimately resulting in increased nutrient transport into the mammary gland[70] and stimulated milk protein production and secretion.[71-72] Preliminary evidence indicates that PRL stimulates both Zip3 and ZnT-4 mRNA expression, although changes in ZnT-4 expression appear to be transient, and inhibition of either JAK/STAT or MAPK signalling pathways using chemical antagonists results in decreased expression suggesting that these pathways somehow participate in the regulatory control of Zn transport mechanisms. A more convoluted question is: how does PRL stimulation result in the movement of Zip3 and ZnT-4 from one cellular location to another? We have preliminary evidence that indicates that both Zip3 and ZnT4 are themselves phosphorylated and studies are currently underway to determine if this phosphorylation is altered by PRL exposure.

5. CONCLUSION

In summary, using the lactating rat as a model we have determined that milk Zn, Cu and Fe levels are regulated temporally through coordinated changes in gene expression, protein levels and localisation of mineral-

specific transporters. While milk Zn, Cu and Fe levels remain somewhat refractory to maternal trace mineral status, maternal malnutrition may have unique effects on mammary gland mineral transporters through secondary effects on hormonal signalling in the mammary gland.

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