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ORIGINAL ARTICLE

GLUT1 and GLUT8 support lactose synthesis in Golgi of murine mammary epithelial cells

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Abstract

The mammary gland increases energy requirements during pregnancy and lactation to support epithelial proliferation and milk nutrients synthesis. Lactose, the principal carbohydrate of the milk, is synthetized in the Golgi of mammary epithelial cells by lactose synthase from glucose and UPD galactose. We studied the temporal changes in the expression of GLUT1 and GLUT8 in mammary gland and their association with lactose synthesis and proliferation in BALB/c mice. Six groups were used: virgin, pregnant at 2 and 17 days, lactating at 2 and 10 days, and weaning at 2 days. Temporal expression of GLUT1 and GLUT8 transporters by qPCR, western blot and immunohistochemistry, and its association with lactalbumin, Ki67, and cytokeratin 18 within mammary tissue was studied, along with subcellular localization. GLUT1 and GLUT8 transporters increased their expression during mammary gland progression, reaching 20-fold increasing in GLUT1 mRNA at lactation ($p < 0.05$) and 2-fold at protein level for GLUT1 and GLUT8 ($p < 0.05$ and 0.01, respectively). The temporal expression pattern was shared with cytokeratin 18 and Ki67 ($p < 0.01$). Endogenous GLUT8 partially co-localized with 58 K protein and α-lactalbumin in mammary tissue and with Golgi membrane–associated protein 130 in isolated epithelial cells. The spatial-temporal synchrony between expression of GLUT8/GLUT1 and alveolar cell proliferation, and its localization in *cis*-Golgi associated to lactose synthase complex, suggest that both transporters are involved in glucose uptake into this organelle, supporting lactose synthesis.

Keywords GLUT8 · GLUT1 · Lactose · Golgi · Mammary cells

Introduction

The mammary gland is an organ composed of a stroma rich in adipose cells and a glandular epithelium that develops into a lobule-alveolar system with alveolar epithelial cells involved in milk production in its terminal zone. This gland undergoes cyclic changes during the female fertile life, reaching its maximal development in lactation, finishing with its involution after weaning, repeating the cycle in each pregnancy until menopause. The mammary gland is used as a unique model of morphogenesis in adults with four defined steps: (1) the proliferative phase, that starts with conception and is associated with a high rate of epithelial cell proliferation, development of ducts and acini; (2) the secretory differentiation phase, in the first step of lactation, when there is activation of enzymes involved in lipid synthesis; (3) the secretory activation phase, after delivery; and (4) the lactation phase, when the continuous secretion of milk is established [3]. There are, however, some inter-species differences. For example, the proliferative phase ceases at the middle of lactation in humans, meanwhile persists during all lactation period in rodents and

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maintains a minimal rate of 0.3% until lactation peak in ruminants [10].

The supply of glucose to the mammary gland is pivotal to maintain the high rate of proliferation of glandular epithelium in pregnancy and the continuous production of lactose (beta 1,4-galactoglucose), fatty acids, and proteins during lactation. The Golgi of mammary epithelial cells (MEC) is unique in its necessities of glucose, because it is the place of lactose synthesis from UPD galactose and glucose. Lactose synthase is a proteic complex composed of galactosyl transferase and lactalbumin (LALB). LALB itself is secreted through the milk together with lactose, triacylglycerides, and proteins like α, β, κ caseins and β-lactoglobulin [1, 10]. Lactose content in the milk is around 5% in all species, which reveals that its synthesis is a highly conserved process. Lactose is also an osmotically active molecule, defining water milk content up to 80% [1, 29].

Glucose transporters already identified in MEC are glucose facilitative transporters GLUT1, GLUT8, and GLUT12; SGLT1; and the bidirectional sugar transporter SWEET1, located in plasma membrane and Golgi [19, 22, 31]. There are reports of increased expression of those transporters in pregnancy and/or lactation in different models, including rodents and ruminants, but it is not clear their involvement in glucose uptake at Golgi of MEC. With the aim of defining the role of GLUT1 and GLUT8 glucose transporters in lactose synthesis, in this study, we examined the temporal and spatial association between the expression of both transporters with LALB in mammary gland during pregnancy and lactation, studying complementarily its association with Ki67 expression. Our results lead us to conclude that GLUT8 and GLUT1 are localized at *cis*-Golgi of MEC and share the same temporal expression pattern of LALB, Ki67, and cytokeratin 18 (CK18), associating lactose synthesis with MEC expansion and proliferation.

Methodology

Experimental procedures

Mammary tissue was obtained from inguinal and abdominal glands of primiparous female BALB/C mice anesthetized with a sodium pentobarbitone solution (2 mg/kg weight) and euthanized with anesthetic overdose after surgical procedure. Six groups of 5 females were used: virgin, pregnancy at 2 and 17 days, lactation at 2 and 10 days, and weaning at 2 days [18]. Day zero of pregnancy was considered when the vaginal plug was observed. At parturition, animals were placed individually, adjusting litters to 6 pups. Animals were maintained at 22 °C with light/dark inverted cycle, feeding ad libitum until the time of procedure. The Institutional Ethic Committee approved all procedures performed in animals conformed to the Guide of the Care and Use of Laboratory Animals of the National Council for Science and Technology Research (CONICYT, Chile).

Isolation and culture of MEC Mammary epithelial cells were isolated from inguinal mammary glands of mice at 2 days of lactation after collagenase digestion [27] and cultured in Dulbecco modified medium supplemented with 10% fetal bovine serum, 1% mammary growth supplement (MEGS), and antibiotic at 5% CO_2. For immunofluorescence assays, cells were plated in circle glasses over 24 well plates at 5×10^4 cells/plate. After 24 h, cells were fixed and processed according to previous protocol [20].

Western blot Mammary tissue (3 g) or 2×10^6 cultured MEC were snap frozen in liquid nitrogen, homogenized in a Polytron with ice-cold radioimmunoprecipitation assay buffer plus protease inhibitor cocktail (Sigma-Aldrich), and stored at − 80 °C until used [18]. Immunoblots were performed on 20 μg of immobilized protein in polyvinylidene fluoride membrane (Hybond-C, Amershan) and incubated overnight with the indicated primary antibody (Supplementary Table I) before revealing with a chemiluminiscent method (BioRad). Values were normalized respect to actin and expressed as increasing over virgin group. LALB was expressed over expression at 17 days of pregnancy.

RT-qPCR Total RNA was obtained from mammary tissue using Absolutely RNA Miniprep Kit (Agilent Technologies). The absorbance ratio > 2.0 at 260/289 and absence of 28S and 18S ribosomal RNA band degradation were used as indexes of purity and integrity of the RNA samples, respectively [26]. Quantitative real-time PCR amplification was done in triplicate on an AriaMx real-time PCR system (Agilent) using 100 ng of RNA as template, 200 nM of each primer and Brilliant II SYBR Green QRT-PCR 1-step Master mix (Agilent Technologies) according to manufacturer guidelines, analyzing data by ddT method [17]. The optimal annealing temperature and magnesium concentration were optimized for each primer pair using RNA prepared from two positive controls (colon and testis). The specificity of the qPCR products was verified with the dissociation curve analysis of the MxPro qPCR software (evidence of a singctrophoresis fractionation in 2% agarose gel of random samples (evidence of a simple amplification product of the expected length for each primer pair, Supplementary Fig. 4). A list of primers pair can be found in Supplementary Table II.

Immunohistochemical assays Assays were performed in 5-μm slides of paraffin-embedded samples using antibodies anti-GLUT1, GLUT8, and antigen Ki67, revealed with respective horseradish peroxidase-coupled secondary antibodies and 2,2-diaminobenzydine (Supplementary Table I), and counterstained with hematoxylin. Positive control tissues were colon for GLUT1 and Ki67, and testis for GLUT8; negative controls were performed in absence of primary antibody. Motic Image Pro 2.0 software with Motic Ba 410 microscope was used to photograph representative peroxidase staining samples at × 40 and × 100 magnification.

Immunofluorescence assays Subcellular co-localization of GLUT8 transporter with LALB and 58 K Golgi protein (58 K) were performed in paraffin-embedded tissue sections, meanwhile its co-localization with Golgi membrane–associated protein 130 (GM130) was performed in cultured MEC, revealed with the respective secondary antibodies conjugated with Alexa-Fluor 594 or Alexa-Fluor 458 (Supplementary Table I). Samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and negative controls were performed in absence of primary antibody. Olympus IX81 fluorescence microscope with a Hamamatsu ORCA-R2 camera controlled by Olympus Xcellence R software was used to photograph representative fluorescence samples at × 60 and × 100 magnification.

Statistical analysis Statistical analyses were performed by one-way ANOVA with Dunnett's post-hoc Tukey test. Each condition was compared to virgin group using the software Graph Pad Prism 6.0, p values < 0.05 were consider statistically significant (*) and < 0.01 highly significant (**).

Results and discussion

GLUT8 and GLUT1 increase their expression in mammary gland during lactation in association with expansion of MEC

We found that GLUT1 mRNA expression increased 23.0 ± 9.0 at 10 days of lactation ($p > 0.01$) (Fig. 1a). Its increase was lower at protein level, reaching only 2.5 ± 0.3-fold virgin group at day 2 of lactation ($p < 0.01$). On the other hand, we assessed not significant changes in GLUT8 mRNA expression, but at protein level GLUT8 increased 2.2 ± 0.3 at day 10 of lactation ($p < 0.05$) (Fig. 1b). The apparent incongruence between GLUT1 mRNA and protein levels can be explained by differential translation regulation, decreased

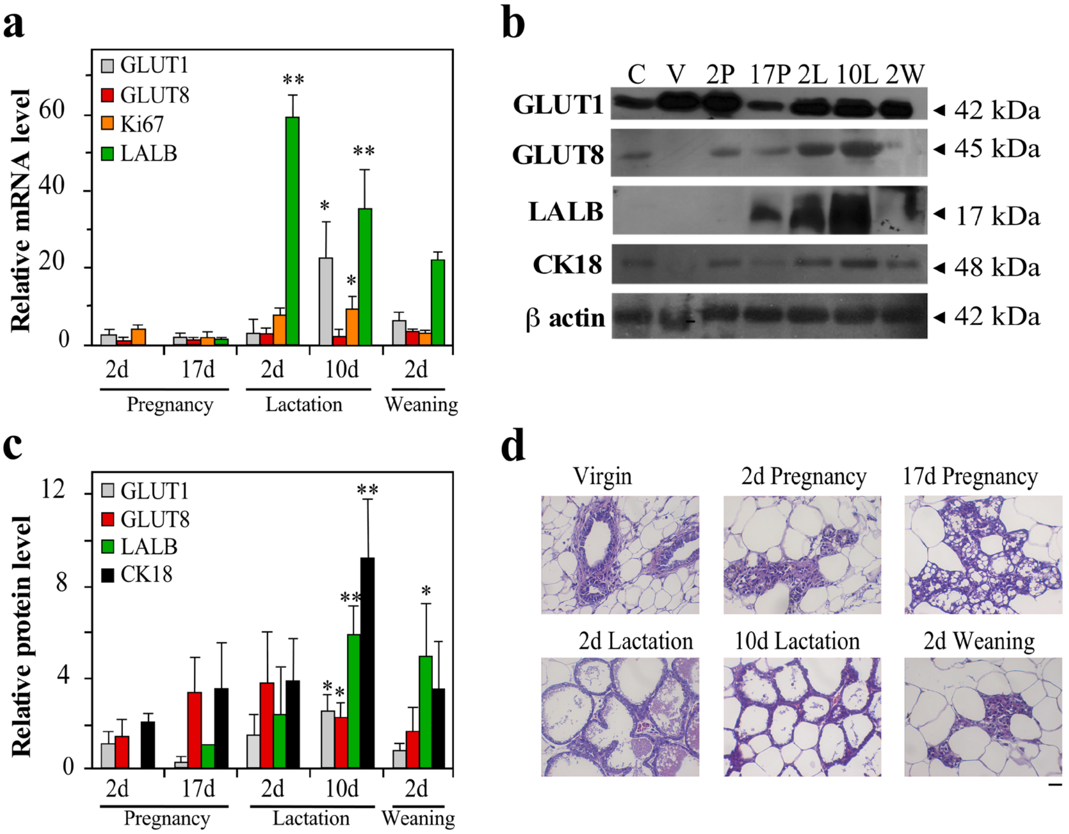

Fig. 1 **a**. Relative abundance of GLUT1, GLUT8, LALB, and Ki67 mRNA by qPCR; data was normalized with respective β-actin and cyclophilin A expression and virgin group. **b**. Western blot of GLUT1, GLUT8, LALB, CK18, and β-actin. Positive control: colon to GLUT1 and testis to GLUT8, CK18, and β-actin. **c**. Densitometric analysis of western blots; data was normalized with respective β-actin expression and virgin group. **d**. Hematoxylin-eosin staining. Bar 50 μm. In **a** and **c**, the results were expressed as mean ± standard deviation of four samples by group, *$p < 0.05$, **$p < 0.01$. The images in **b** and **d** correspond to representative results of four independent samples by group

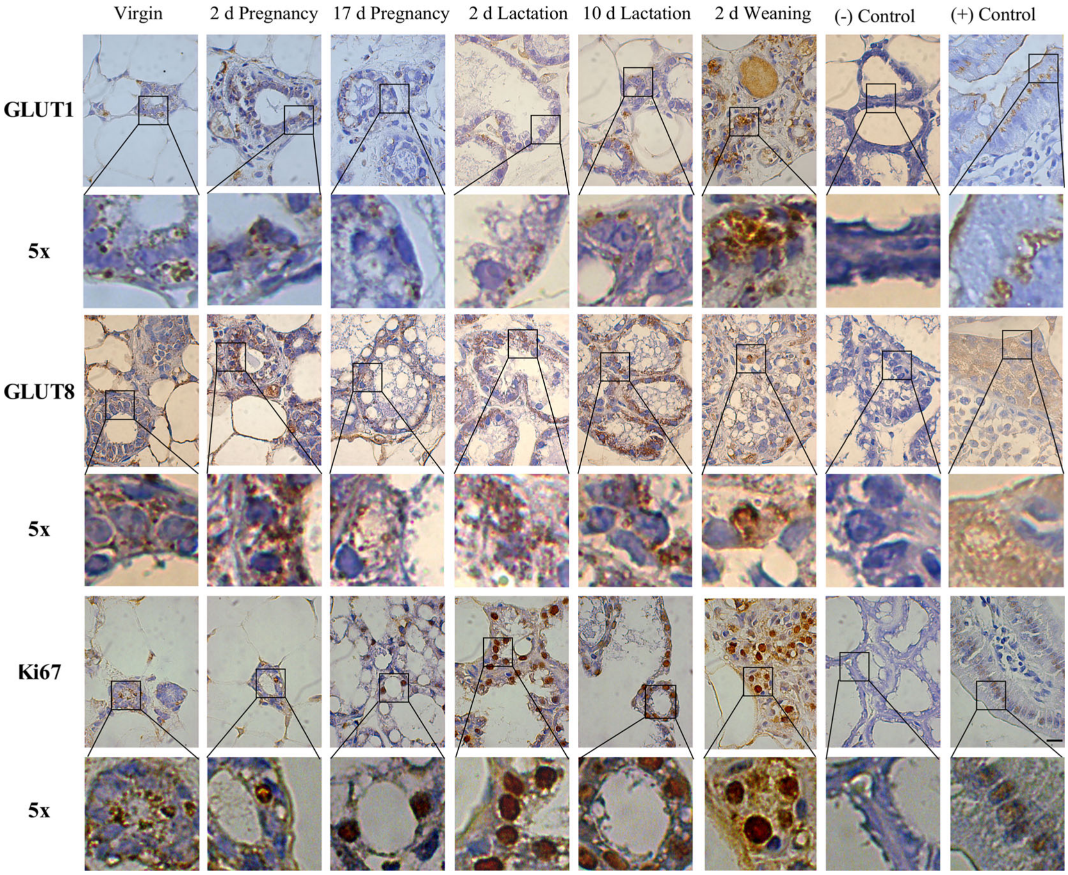

Fig. 2 GLUT1, GLUT8, and Ki67 immunohistochemistry in mammary gland at pregnancy, lactation, and weaning. Bar 20 μm

mRNA stability or expression of miRNA, an issue that has not been explored. Supporting the last possibility, Alsaweed et al. reported the presence of high GLUT1 miRNA level in human milk, which could decrease its translational rate [2]. The pattern of glucose transporters expression that we found here is in concordance with previous reports, although with some differences in magnitude. For example, GLUT1 mRNA increases 50-fold at lactation peak in ruminants and 10-fold in rodents; and GLUT8 mRNA increases 2-fold in rodents [30]. Higher GLUT1 expression in cows could be associated to a high secretory activity of MEC from ruminants [6]. Underlying factors responsible for GLUT1 upregulation in mammary gland at lactation are serotonin, hypoxia, growth hormone, and lactogenic hormones, but no data about GLUT8 regulation is reported [30]. GLUT12 and SGLT1 are other glucose transporters whose expression increases during lactation, but with lower magnitude than it is shown here for GLUT1 and GLUT8 [30, 32].

We also found that mRNA levels of LALB mRNA increased suddenly at day 2 of lactation to 58.4 ± 9.9 ($p > 0.01$) (Fig. 1a). At protein level, LALB also appears at late pregnancy, increasing to 5.8 ± 1.3-fold ($p < 0.01$) at day 10 of lactation, remaining elevated at weaning (4.9 ± 2.3, $p < 0.05$), meanwhile CK18 increases 9.2 ± 2.9-fold at 10 days of lactation ($p < 0.01$, Fig. 1c). GLUT1 and GLUT8 expression pattern is partially shared with LALB and CK18, revealing a synchrony between glucose transporter expression, milk synthesis, cell proliferation, and epithelial expansion. In accordance with previous reports, we found a ratio of 10:1 during lactation between mRNA and protein for GLUT1 and LALB [30]. The hematoxylin-eosin staining confirms the progressive increase in parenchymal tissue as pregnancy and lactation progress and its involution after weaning. The fat globules in MEC evidence secretory activity of the gland at 17 days of pregnancy and accumulation of protein secretion in the lumen of the acini at 2 days of lactation (Fig. 1c). It is well established that mammary glucose uptake is the rate-limiting step in milk production and that its increase during pregnancy and lactation is associated to increase in glucose transporters expression and MEC proliferation [9, 30]. The low Ki67 mRNA level on virgin and early pregnancy groups could represent stem cell renewing, which does not exceed 5% [6]. Our results indicate that MEC shows Ki67 highest expression at 10 days of lactation in contrast to previous reports that establish a proliferation peak at 5 days of lactation, with duplication every 6 days. It is possible that these differences can arise because in that study proliferation was measured by DNA recording and titrated thymidine incorporation, which are active only replication phase, meanwhile Ki67 expression is maintained elevated during all cell cycle steps and could remain high after cell division [13, 14].

Immunohistochemical assays revealed that GLUT8 and GLUT1 were intracellular and Ki67 nuclear in MEC during all the steps studied, with variable intensity and quantity of stained cells (Fig. 2). For GLUT1, the staining was weak, highlighting the weaning with the highest reactivity and late pregnancy with the least, meanwhile for GLUT8, the behavior was completely different; an evident progressive increase in the reactivity was observed until reaching the highest expression at middle lactation, similarly to Ki67. The almost exclusive intracellular expression of GLUT1 in murine MEC found here is opposed to results of Nemeth et al. in rats, where GLUT1 was at basolateral membrane [22], revealing interspecies difference in MEC glucose transporters localization. In our opinion, in absence of GLUT1 in plasma membrane of MEC, SGLT1 would be enough to support glucose uptake, because, as a secondary active transporter, it mobilizes glucose inside the cell using the sodium electrochemical gradient [30]. The highest expression of GLUT1 in MEC after 2 days of forced weaning could be associated to its accumulation in a proteasomal compartment or apoptotic bodies phagocyted by MEC acting as non-professional phagocytes [3, 4]. Soon after forced weaning, GLUT1 re-localization from plasma membrane to intracellular compartment was observed in other study [22].

cis-Golgi localization of GLUT8 and GLUT1 is consistent with their role in lactose synthesis on MEC

Subcellular localization of GLUT8 and its association with lactose synthase complex in mouse mammary gland was determined by co-localization with Golgi markers in paraffin-embedded tissue and subsequent confocal microscopy analysis. We found that GLUT8 co-localized with LALB and 58 K Golgi protein in lactating mammary gland, but also is expressed in other unidentified intracellular compartment (Fig. 3a). We also explored the subcellular localization of endogenous GLUT8 in cultured MEC isolated from lactating mammary gland where co-localized partially with GM130 antigen (Fig. 3). The co-localization of GLUT8 with LALB, 58 K protein, and GM130 antigen in MEC of mammary tissue reflects its partial localization in *cis* and medial Golgi of MEC, where lactose synthase is localized, revealing a spatial association and probably a functional coupling between glucose uptake and lactose synthesis. In this regard, Laporta et al. reported that endogenous GLUT1 is in plasma membrane of isolated goat MEC, meanwhile GLUT1_GFP was predominantly found in Golgi, revealing changes in localization of over-expressed transporters [15, 16]. Previously, GLUT1 was co-localized with 110-kDa coatomer-associated protein β-COP, another cis-Golgi protein, and studies of cytochalasin B binding identified GLUT1 in Golgi's isolated vesicles from

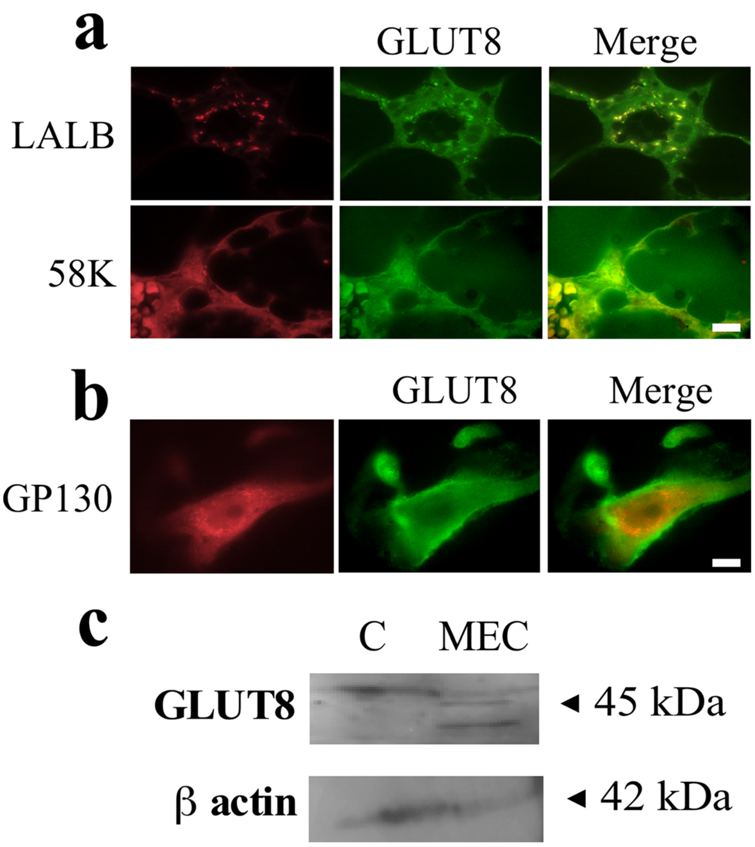

Fig. 3 **a**. Subcellular localization of GLUT8 in mammary gland at 2 days of lactation. **b**. Subcellular localization of GLUT8 in cultured isolated MEC. **c**. Westen Blot of GLUT8 and β-actin expression in cultured isolated MEC. C: positive control, mammary gland, MEC: mammary epithelial cells. Bar 50 μm in**a** and 10 μm in **b**

MEC [19, 22].In relation to GLUT8, it has been localized intracellularly in MEC, at the endomembrane system (reticulum, Golgi, lysosome/endosome) in other cell types and specifically at plasma membrane in some few [5, 11, 12, 24]. Intracellular GLUT8 has been related to protein glicosylation by galactosyl transferase at Golgi, meanwhile its mobilization to plasma membrane had been reported in response to insulin and glucose in trophodermic cells and neurons, respectively [7, 23, 24]. However, Augustin et al. discarted the presence of GLUT8 at the recycling vesicle pool in several stable transfected cell lines, which is indicative of it essential role as intracellular transporter [5]. Moreover, dileucine motif ($LL^{12/13}$) and interaction with adaptin AP-2 and dynamin GTPase are responsible of GLUT8 lysosomal localization [25]. In resume, GLUT8 seen to be a complex multifunctional glucose transporter, whose specific function and localization vary depending on the cell type. Particularly in MEC, GLUT8 would support glucose transport for lactose synthesis at Golgi. Will be interesting to study possible regulatory factors that changes GLUT8 expression or subcellular localization in lactating mammary gland, including transcription factors.

Association of GLUT1 and GLUT8 expression at Golgi of mammary epithelial cells with glucose transport and lactose synthesis

Although there are data about intracellular expression of GLUT1/GLUT8 transporters in MEC, the only functional studies of glucose transport in Golgi of mammary gland were published long time ago using osmotic lysis and inhibition of lactose synthesis [28, 32]. Those studies revealed that GLUT and SGLT transporters are expressed in Golgi of MEC, because specific inhibitors of both glucose transporters affected the assays. The differential permeability to carbohydrates observed in Golgi's vesicles suggests that GLUT transporters are predominant, specifically GLUT8, due to the higher permeability to fructose than to mannose or galactose, because GLUT1 does not transport fructose. On the other hand, the low permeability to disaccharides in Golgi's vesicles could be indicative of low SWEET1 transporter expression, which also mediates glucose efflux when is expressed at plasma membrane [32]. Also, the quantitative western blot assay performed in Golgi's vesicles before that GLUT8 was discovered is indicative that GLUT1 and GLUT8 transporter are expressed at the Golgi of MEC [19]. There is evidence that sodium-vitamin C cotransporter 2 decreases 600-fold its affinity at intracellular ionic concentration (low sodium and high potassium) and that GLUT1, a bidirectional transporters is asymmetric, showed 10-fold higher $V_{\max}$ for exit than for entry at low temperature [8, 21]. It is possible that GLUT8 expressed at Golgi of MEC presents different kinetic proprieties than the values reported when it is expressed at plasma membrane or that this transporter has asymmetric glucose transport as GLUT1. Both issues are not yet explored and could be interesting to study in relation with MEC's Golgi glucose uptake.

Concluding remarks

The temporal expression of GLUT1 and GLUT8 and its location in Golgi of mammary cells suggest that they are involved in glucose intake of this organelle, supporting lactose synthesis. Moreover, they share their temporal pattern with alveolar cell's proliferation and LALB expression, increasing their expression principally in lactation. This study opens up new lines of research on the need to define the factors responsible for GLUT8 upregulation during lactation as well as its function as Golgi glucose transporter.

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Compliance with ethical standards

The Institutional Ethic Committee approved all procedures performed in animals conformed to the Guide of the Care and Use of Laboratory Animals of the National Council for Science and Technology Research (CONICYT, Chile).

Conflict of interest The authors declare that they have no conflict of interest.

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