Regulation of gene expression in human mammary epithelium: effect of breast pumping

Patricia D Maningat, Partha Sen, Agneta L Sunehag, Darryl L Hadsell and Morey W Haymond

Department of Pediatrics - Nutrition, Baylor College of Medicine, Children's Nutrition Research Center # 7062, 1100 Bates, Houston, Texas 77030, USA

(Correspondence should be addressed to M W Haymond; Email: mhaymond@bcm.tmc.edu)

Abstract

Little is known of the molecular regulation of human milk production because of limitations in obtaining mammary tissue from lactating women. Our objectives were to evaluate whether RNA isolated from breast milk fat globules (MFGs) could be an alternative to mammary biopsies and to determine whether intense breast pumping, which increases prolactin (PRL) secretion, will upregulate α -lactalbumin (α -LA, a major determinant of lactose synthesis) transcription. RNA was isolated from MFG and transcripts of interest were identified and quantitated by real-time RT-PCR using an external standard for normalization. In addition, we performed microarray studies to determine MFG RNA gene expression profile. Ten lactating women were studied using two protocols: protocol A with intense pumping from 0800 to

0814 h followed by short pumping and protocol B with intense pumping from 1200 to 1214 h preceded by short pumping. Plasma PRL and MFG α -LA mRNA expression were measured. During protocol A, plasma PRL (61 ± 7 – 248 ± 43 $\mu\text{g/l}$ by 14 min) and α -LA (3.5 ± 0.9 fold by 6 h; $P < 0.03$) increased. During protocol B, PRL gradually increased over 4 h from 69 ± 14 to 205 ± 28 $\mu\text{g/l}$, and further to 329 ± 23 $\mu\text{g/l}$ by 12 min of intense pumping; α -LA mRNA expression did not increase significantly. We conclude that MFGs provide a unique source to study the *in vivo* regulation of gene expression in mammary epithelial cells. α -LA mRNA is abundant in the MFG and its expression may be regulated by hormonal and temporal factors.

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Introduction

The establishment and maintenance of lactation require the concerted regulation by hormones, genes, and local factors. However, little is known about the molecular regulation of milk production in humans due to limitations in obtaining mammary tissue. Therefore, a non-invasive technique permitting the analysis of gene expression in mammary epithelial cells would provide insight into the complex processes of milk production in humans.

Milk fat is secreted by an apocrine mechanism unique to mammary epithelial cells (McManaman *et al.* 2004). Cytoplasmic lipid droplets are enveloped in plasma membrane, and pinched off, resulting in the secretion of milk fat globules (MFGs). Intracellular components of the mammary epithelial cells become trapped within the MFG as a cytoplasmic crescent containing cytoplasmic material except the nucleus (Huston & Patton 1990). Thus, the MFGs represent a potential source to repetitively sample the cytoplasm of mammary epithelial cells.

α -Lactalbumin (α -LA), the regulatory subunit of the lactose synthetase enzyme complex, is abundantly expressed *de novo* only in the epithelial cells of the mammary gland (Lo *et al.* 1998) and drives this enzyme complex to synthesize lactose (Jagoda & Rillema 1991, Stacey *et al.* 1995, Ramakrishnan *et al.* 2001). Lactose, in turn, is the primary

osmotic agent in milk and, therefore, is thought to be the primary determinant of milk volume.

We report here a non-invasive method for the isolation and quantitation of RNA from human MFG, the steps taken to validate its specificity for the mammary epithelial cell, and the steps to find a reference gene. Quantitative real-time reverse transcription PCR (QRT-PCR) was used to assess gene expression in the MFG RNA; and microarray studies were utilized to examine the MFG RNA transcriptome. We have utilized this new technique to test the hypothesis that increased plasma PRL resulting from a period of intense pumping will upregulate α -LA mRNA expression.

Materials and Methods

Subjects

All women were healthy, 18–35 years old, between 6 and 12 weeks *post partum*, had singleton uncomplicated pregnancies and delivered at term (≥ 37 weeks). Their infants were healthy and being exclusively breastfed at the time of the study. Written consent was obtained from each woman prior to enrollment.

Milk samples

Milk samples were obtained from women participating in several lactation protocols (for method development and validation) and in our specific breast pumping protocol; all of which were approved by the Institutional Review Board for Human Subject Research and the Scientific Advisory Committee of the General Clinical Research Center (GCRC) at Baylor College of Medicine (Houston, TX, USA). Breast milk was collected simultaneously from both breasts into sterile milk bottles (Ross Products, Columbus, OH, USA) using a standard breast pump (Playtex Embrace, Dover, DE, USA). Milk bottles were weighed before and after milk collection on a Mettler AE50 balance (Mettler-Toledo, Greifensee, Switzerland) and kept on ice prior to the MFG collection.

Collection of MFGs

Ten milliliters of milk was transferred into sterile, RNase-free tubes, tightly sealed, and then centrifuged (Sorvall Legend T, Germany) at 2680 *g* for 10 min at 4 °C. The supernatant fat layer was transferred using a sterile spatula to a new tube. One milliliter of Trizol (Invitrogen Life Technology) was added prior to storing at −80 °C for samples that were used for the Trizol method of isolation.

Total RNA isolation and assessment of RNA integrity

RNA isolation was performed initially by the RNeasy method (Qiagen Inc.) but later changed to the Trizol method utilizing the manufacturers' protocols. RNA concentration was measured by reading the absorbance at 260 nm using a ThermoSpectronic (Biomate 3; Rochester, NY, USA) spectrophotometer (Manchester 1995). RNA integrity was assessed by standard denaturing 1% Seakern ME agarose (Lonza, Basel, Switzerland) gel electrophoresis (Ausubel 1988, Brown *et al.* 2004) using 5 µg MFG RNA with 1× 3-(N-morpholino) propanesulfonic acid (MOPS) running buffer for 30 min at 80 V in a Bio-Rad MiniSub Cell gel box (Bio-Rad).

Identification of select gene transcripts in MFG by real-time RT-PCR

RNA isolated from random milk samples from two women and a human T-cell line (0.1–10 ng) was used to determine the expression of selected genes of interest by one-step RT-PCR: α -LA, cytosolic phosphoenolpyruvate carboxykinase 1 (PCK1), mitochondrial PCK2, ribosomal 18S (R18S), and colony-stimulating factor 1 (CSF1). Primers for α -LA (cataatgtgtgccaa-gaagatcct and gccactgttcacgttcctcagt) were synthesized in our laboratory, while the probe (6FAMtgactactgtgtggccca-taaagccctctTAMRA) was synthesized by Applied Biosystems (ABI, Foster City, CA, USA). Primer–probe pairs for PCK1 and PCK2 (Assay ID Hs00159918_m1 and Hs00356436_m1 respectively), CSF1 (Hs00174164_m1), and R18S (Hs9

9999901_s1), a candidate reference gene, were purchased from ABI. Reverse transcription (48 °C for 30 min) and PCR cycling parameters were performed following the instruction manual of the ABIPRISM 7900 (95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min, 40 cycles). Reactions were carried out in a 96-well plate in a final reaction volume of 50 µl using TaqMan One Step PCR Master Mix Reagents Kit (P/N 4309169, ABI).

Microarray analysis

Microarray analysis was performed to evaluate the gene expression profile of the MFG RNA from a single subject enrolled in a lactation protocol where breast milk was collected every 3 h (q3h) for 96 h. After the first 24 h of milk collection, 0.1 mg/kg rhGH (Nutropin AQ, Genentech Inc., South San Francisco, CA, USA) was administered subcutaneously at 0830 h for the next 2 days. For initial analysis, 16 samples from different time points were used. Total RNA (1.5 µl) was quantified using the Nanodrop ND-1000 u.v.–vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and the RNA quality was assessed using the Experion RNA StdSens Analysis Kit (Bio-Rad Laboratories) on a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Gene expression analysis was performed using the Sentrix BeadChip and BeadStation system from Illumina Inc. (San Diego, CA, USA). Total RNA between 300 and 500 ng was converted to cDNA by reverse transcription using Superscript II reverse transcriptase and T7-(dT)24 primers, followed by second-strand synthesis to generate double-stranded cDNA. After purification, the cDNA was converted to biotin-labeled cRNA, hybridized to a human Ref-8 BeadChip array (Illumina Inc.), and stained with streptavidin–Cy3 for visualization. The human Ref-8 BeadChips contain sequences representing ~22 000 curated genes and ESTs. Quality standards for hybridization, labeling, staining, background signal, and basal level of housekeeping gene expression for each chip were verified. After scanning the probe array, the resulting image was analyzed using the BeadStudio software (Illumina Inc). Samples were normalized using a rank invariant procedure to the baseline sample. Selected genes, casein (CSN3 and CSN2), xanthine dehydrogenase (XDH), growth hormone receptor (GHR), insulin-like growth factor (IGF1), and IGF1 receptor (IGF1-R), were chosen and the fluorescence intensity data (extracted using the manufacturer's software) were used to determine the expression of these genes. We then used QRT-PCR to confirm the array results for GHR, IGF1, and IGF1-R using the MFG RNA samples from the same subject (10–4000 ng) and compared this with α -LA expression (10–100 ng). Primer–probe pairs were obtained from ABI (Hs00174872_m1, Hs01547656_m1, and Hs00181385_m1 respectively).

Evaluation of candidate reference genes for quantitative real-time RT-PCR

Human R18S RNA The MFG RNA from four subjects was used to determine R18S expression at different time

points in a lactation protocol. One-step QRT-PCR was performed in duplicate 50 µl reactions using 10 ng MFG RNA. R18S expression was calculated as the fold change relative to the baseline sample: fold difference = $2^{-\Delta C_t}$ (Livak & Schmittgen 2001).

Human endogenous control array TaqMan Low Density Human Endogenous Control Array (ABI) was used to determine the expression of 16 commonly used reference genes. Briefly, we reverse-transcribed 100 ng MFG RNA from one subject (taken at five time points in a lactation protocol) using the Retroscrip Protocol (Ambion, Austin, TX, USA). Sixty nanograms of the resulting cDNA product were mixed with Universal Master Mix (P/N 4304437, ABI) and loaded into the array. The array was centrifuged briefly, sealed, and then loaded into the ABIPRISM 7900 using the manufacturer's recommended cycling parameters. For each gene, the mRNA level was expressed as a fold change relative to the baseline sample (see above).

External standard To find a suitable normalizer, we added 10 ng mouse (Mm) lung RNA (Ambion, Cat. No. AM7817) to each 10 ng MFG RNA sample. The resulting Mm lung + MFG RNA mix was used to determine Mm β-actin expression. Mm β-actin gene expression assay (Assay ID Mm00607939_s1) was purchased from ABI. Cycling parameters and calculations were identical to those described for R18S. Serial dilutions of the MFG RNA with equal amounts of Mm lung RNA (0.1–100 ng) were used to evaluate the PCR amplification efficiency of the Mm β-actin and α-LA primer pairs. To determine the relative efficiency of each primer pair, the log of input amount of RNA was plotted against the difference in cycle threshold (C_t), $C_{t_{\alpha-LA}} - C_{t_{Mm\beta-actin}}$.

Breast pumping protocol: effect of increased PRL on α-LA expression

Subjects Ten healthy lactating women ($n=5$ protocol A and $n=5$ protocol B) were included. Subjects were 27 ± 1 (protocol A) and 26 ± 2 (protocol B) years old, weighed 67 ± 3 and 69 ± 6 kg respectively and had a BMI of 26 ± 1 and 25 ± 2 kg/m² respectively.

Study design The subjects were instructed to consume a standard diet (50–60% carbohydrate, 15% protein, and 30–35% fat) 1 week prior to the study. The subjects and their infants were admitted to the GCRC of the Texas Children's Hospital on the afternoon prior to the study. They were given supper (10 kcal/kg) at 1800 h and a light snack at 2100 h, with a similar macronutrient distribution as above. Following the evening snack, the subjects were fasted until the end of the study the next day (1600 h for protocol A and 1800 h for protocol B). At 2200 h on the day of admission, an i.v. line was inserted into the antecubital vein under Ela-Max cream analgesia (Ferndale Laboratories, Ferndale, MI, USA) and

infused with 0.9% NaCl at low rates to keep the vein open for blood sampling. Each woman breastfed her infant on both breasts at 0400 h, and the infant was weighed before and after breastfeeding using an electronic scale (Scale-tronix Pediatric Scale 4802, Wheaton, IL, USA) to determine the volume of milk ingested (Borschel *et al.* 1986). No further breastfeeding was performed until the end of the study.

Protocol A: intense breast pumping at 0800 h Starting at 0800 h, seven successive 2-min milk collections were performed. In addition, one 2-min milk collection was obtained at 1000 h and then q30 min from 1200 to 1600 h. The milk was immediately placed on ice and processed for collection of MFG.

Protocol B: intense breast pumping at 1200 h This protocol was identical to protocol A, except that 2-min breast milk samples were collected q30 min from 0800 to 1200 h, prior to the seven successive 2-min breast milk collections from 1200 to 1214 h. Additional 2-min samples were collected at 1400, 1600, and 1800 h.

Blood (2.5 ml) was collected q30 min from 0400 h until the end of the study, except during the intense pumping, where blood was collected q2 min for measurement of plasma PRL. Subjects were discharged between 1800 and 2000 h after consuming a light snack.

α-LA quantitation One-step QRT-PCR using Mm β-actin as a reference gene was used to quantitate α-LA in the MFG RNA. The expression of α-LA was calculated as the fold change relative to the baseline sample (0800 h) according to the $\Delta\Delta C_t$ method (Pfaffl 2001).

Plasma assays Blood samples (2.5 ml) were centrifuged at 2680 g for 10 min at 4 °C. The plasma was separated and transferred to a new tube then stored at –80 °C. Plasma PRL was measured using an electrochemiluminescence assay (Elecsys 1010, Roche Diagnostics). The inter-assay mean $\pm$ s.d. for the assay control was 7.6 ± 0.7 mg/l with a coefficient of variation (CV) of 9%, while the intra-assay CV among ten replicates was 0.9%.

Statistical analysis

All data are presented as mean $\pm$ s.e.m. Data were compared using Student's *t*-test and ANOVA followed by Tukey's HSD *post hoc* analysis using SPSS for Windows (Version 10.0, SPSS Inc. Chicago, IL, USA).

Results

Total RNA isolation and assessment of RNA integrity

Total RNA yield with the RNeasy method was 3.9 ± 0.4 µg/ml breast milk, while that using the Trizol method was 4.5 ± 0.4 µg/ml breast milk ($P=NS$). RNA isolated

Figure 1 (A). Microarray studies demonstrated high fluorescence intensity for the milk protein-related genes: α -lactalbumin (α -LA), casein 2 (CSN2), casein 3 (CSN3), and xanthine dehydrogenase (XDH). (B) Insulin-like growth factor receptor (IGF1-R) was expressed at a much lower fluorescence intensity, while growth hormone receptor (GHR) and insulin-like growth factor (IGF1) were not detected. Fluorescence values (normalized to baseline sample) are the mean $\pm$ S.E.M. of 16 MFG RNA samples obtained over 4 days of study from a single subject. Note different scales on y-axis.

using Trizol method had significantly less RNA degradation when compared with the RNeasy method (data not shown). Therefore, all subsequent RNA isolations were done by the Trizol method.

Identification of select gene transcripts in MFG by real-time RT-PCR

α -LA, a milk protein gene, was only detected in the MFG RNA but not in the RNA isolated from T cells. PCK1 was not detectable, while PCK2 was detected in both MFG and T-cell RNA, albeit in low amounts. CSF1 (marker of

macrophage contamination) expression was not detected in the MFG RNA. R18S was expressed in high amounts in both MFG and T-cell RNA, thus we proceeded to validate this as a reference gene (see below).

Microarray analysis

Based on the fluorescence intensity data, milk protein-related genes such as CSNs, α -LA, and XDH were highly expressed. IGF1-R was expressed but at a much lower level than the milk protein-related genes, while GHR and IGF1 were not detectable (Fig. 1). RT-PCR confirmed the array results: GHR and IGF1 were not detectable (C_t of 40) in up to 2 μ g MFG RNA, while IGF1-R was consistently expressed at low levels when using 1–4 μ g RNA (Table 1).

Evaluation of candidate reference genes for quantitative real-time RT-PCR

Ribosomal 18S R18S expression in MFG RNA varied greater than fivefold over the time course of the samples analyzed (data not shown) and was deemed unsuitable as a reference gene.

Human endogenous control array Among the candidate genes in the array, R18S was found to be the most highly expressed but varied considerably across time, confirming our initial real-time PCR results using R18S primers (see above). The expression of other endogenous reference genes varied by as much as 16-fold from the baseline, but followed the same expression pattern. As a result, they were deemed unsuitable as reference genes for the quantitation of α -LA (Fig. 2).

Mouse β -actin Using the external standard, fold change of Mm β -actin expression was 1.6 when compared with the baseline (Fig. 3). The intra-assay variability was determined using ten replicates of 10 ng MFG RNA + 10 ng Mm lung RNA. The average C_t for Mm β -actin was 25.10 ± 0.04 (mean $\pm$ S.E.M.) and the coefficient of variation was 0.2% (data not shown). The slope for the plot of the log input (0.1–100 ng) RNA versus ΔC_t ($C_{t\alpha-LA} - C_{tMm \beta-actin}$) was 0.11, demonstrating comparable amplification efficiencies over the assessed range

Table 1 Cycle thresholds for specified genes in human milk fat globule (MFG) RNA. Data presented as mean $\pm$ S.E.M.

Gene	Total MFG RNA (ng)				
	10 ng	100 ng	1000 ng	2000 ng	4000 ng
α -LA	32.7 $\pm$ 1.4	29.4 $\pm$ 1.6	NT	NT	NT
IGF1-R	40	40	36.5 $\pm$ 0.7	35.5 $\pm$ 0.3	34.3 $\pm$ 0.2
IGF1	40	40	40	40	NT
GHR	40	40	40	40	NT

Real-time PCR cycle thresholds (C_t) for detection of α -lactalbumin (α -LA), growth hormone receptor (GHR), insulin-like growth factor (IGF1), and insulin-like growth factor receptor (IGF1-R) in varying concentrations of MFG RNA. A C_t of 40 means no detection. The PCR results for these genes support the microarray data (Fig. 1B). $n=4$. NT, not tested.

Figure 2 The expression profile of 16 potential reference genes in the TaqMan Human Endogenous Control Array. Data are shown as the fold change from baseline. None of these genes were suitable to be used as a reference gene for normalization. R18S, ribosomal 18S; ACTB, β -actin; B2M, β 2-microglobulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GUSB, β -glucuronidase; HMBS, hydroxymethylbilane synthase; HPRT1, hypoxanthine phosphoribosyltransferase 1; IPO8, importin 8; PGK1, phosphoglycerate kinase 1; POLR2A, polymerase (RNA) II (DNA-directed) polypeptide A; PPIA, peptidylprolyl isomerase A (cyclophilin A); RPLPO, human acidic ribosomal phosphoprotein PO; TBP, TATA binding protein; TFRC, transferrin receptor; UBC, ubiquitin c; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein.

(Suslov & Steindler 2005). Thus, the Mm β -actin was suitable as a reference gene for $\Delta\Delta C_t$ quantitation.

Effect of breast pumping on prolactin (PRL) and α -LA mRNA expression

Protocol A The PRL concentration following breastfeeding at 0400 h was 246 ± 23 μ g/l, gradually fell to a nadir at 0800 h (61 ± 7 μ g/l), and then peaked acutely to 248 ± 43 μ g/l by 14 min after the start of intense pumping. This peak was essentially identical to that observed following the 0400 h breastfeeding, suggesting that similar stimulation of the breast occurred under both study conditions. α -LA mRNA in MFG increased 3.5 ± 0.9 -fold after 6 h ($P < 0.03$), and then returned toward baseline by 8 h (Fig. 4).

Protocol B The PRL concentration after the 0400 h breastfeeding (234 ± 33 μ g/l) declined to 69 ± 14 μ g/l by 0800 h. During the short pulses of q30 min pumping from 0800 to 1200 h, PRL concentration gradually increased to 205 ± 28 μ g/l by 1200 h. Intense breast pumping at noon resulted in a further increase of PRL to 329 ± 23 μ g/l by 12 min, and then declined continuously until the end of the study. Alternatively, we calculated the PRL area under a curve (AUC) from 0400 to 1600 h, 1799 ± 246 μ g/12 h (protocol A) versus 1588 ± 201 μ g/12 h (protocol B), $P = 0.21$, suggesting similar breast stimulation for both protocols. The milk yield during breastfeeding at 0400 h (127 ± 14 ml) was significantly higher than that during the 2-min pumping at 0800 h (24 ± 4 ml), $P = 0.0003$, suggesting

Figure 3 Real-time RT-PCR comparing the expression of some endogenous controls from TaqMan Human Endogenous Control Array (from Fig. 1, panel A) versus an added external standard (panel B). Mouse (Mm) RNA was added to the MFG RNA samples, and the expression of β -actin was quantified using Mm β -actin probes and primers. The addition of a known amount of external standard resulted in more stable expression needed for use as a normalizer in relative quantitation. Data presented as the fold change from the baseline sample. $n=4$, Mean $\pm$ S.E.M. Note: plotted on different scales.

that the breasts were incompletely emptied during the 2-min pumping. During the intense pumping from 1200 to 1214 h, total milk yield was only 22 ± 7 ml. None of the subjects had enough milk to complete the intense pumping collection, and by min 6 of collection, four subjects pumped <1 ml milk per 2-min collection. The α -LA mRNA expression varied between 0.5 and 1.5 times the baseline, but the fold change differences were not significant (Fig. 4).

Discussion

The development of a non-invasive technique for obtaining tissue samples from lactating women would make possible the exploration of molecular mechanisms regulating human lactation. Fat secretion occurs through an apocrine

mechanism and brings with it mammary epithelial cell cytoplasmic material, including ribosomes, mitochondria, and other organelles (Huston & Patton 1990, Lindquist *et al.* 1994, Thompson *et al.* 1998). As much as 38% of the lipid droplets in human breast milk contain cytoplasmic crescents (Huston & Patton 1990). From this cytosol within the MFG, we have been able to consistently isolate ~ 4 μ g total RNA per milliliter of breast milk. Initial efforts at RNA isolation using the RNeasy kit were hampered by RNA degradation, but the quality was substantially improved using the Trizol method, suggesting that degradation by ribonucleases had not occurred in the mammary lacteal prior to milk collection.

DNA and RNA of epithelial cells isolated from human milk have been shown to represent gene expression in the mammary gland (Lindquist *et al.* 1994, Thompson *et al.* 1998, Boutinaud & Jammes 2002). However, breast milk contains a significant number of leukocytes, and 4–44% of cells in milk from women 4 weeks *post partum* are non-epithelial. Thus, additional procedures are needed to isolate mammary epithelial cells prior to RNA isolation (Brooker 1980, Obermeier *et al.* 2000, Boutinaud & Jammes 2002, Boutinaud *et al.* 2002). Since we discarded the pellet that results from centrifugation of the whole milk, we eliminated these other cellular elements. This was confirmed by the absence of CSF1 gene expression (marker for contaminating macrophage RNA).

The demonstration of high α -LA expression in the MFG RNA lends further support that the MFG RNA was representative of the mammary epithelial cell. α -LA is a whey protein found only in mammary epithelial cells and forms the non-catalytic subunit of the enzyme lactose synthetase. α -LA modifies the specificity of the active enzymatic subunit galactosyl transferase, central to the synthesis of lactose. Lactose, in turn, is thought to be the primary osmotic agent in milk. In mice, it has been shown that the absence of α -LA results in failure of lactation, while lactation was restored with the expression of human α -LA gene (Stinnakre *et al.* 1994, Stacey *et al.* 1995). A 170 bp fragment, the size predicted for the human α -LA PCR product, was obtained from MFG RNA (data not shown). This RT-PCR product was not detected in RNA from a T-cell culture line, indicating that the α -LA transcripts were specific to mammary epithelial cells. R18S, as expected, was highly expressed in all RNA samples, PCK1 expression was absent, and PCK2 was detected at low levels. Our results are reminiscent of those observed in guinea pigs where PCK2 (mitochondrial PCK) is the more active isoenzyme during lactation (Jones *et al.* 1989).

In addition, we evaluated the MFG transcriptome by microarray analysis. α -LA, CSNs, and XDH (Fig. 1) genes that are expressed by the mammary epithelial cell (Naylor *et al.* 2005) were highly expressed. The mechanism by which hGH acts on the lactating mammary gland is controversial. Others have been unable to demonstrate specific binding of GH to mammary epithelial cells (Akers & Keys 1984, McFadden *et al.* 1990), and IGF1 mRNA was shown to be expressed by the mammary stroma and not by the mammary

Figure 4 Comparison of PRL levels and α -LA mRNA expression for protocols (A) and (B). Protocol A: intense breast pumping at 0800 h (time 0). PRL levels peaked after 0400 h (time -4) breastfeeding at $246 \pm 23 \mu\text{g/l}$, declined to $61 \pm 7 \mu\text{g/l}$ at 0800 h and then increased acutely to $248 \pm 43 \mu\text{g/l}$ by 14 min after the start of intense pumping; and α -LA mRNA increased 3.5-fold ($P < 0.03$) 6 h from 0800 h. Protocol B: intense breast pumping at 1200 h. PRL increased after 0400 h to $234 \pm 33 \mu\text{g/l}$ (time -4) breastfeeding, declined to $69 \pm 14 \mu\text{g/l}$ at 0800 h, gradually increased to $205 \pm 28 \mu\text{g/l}$ by 1200 h (start of intense pumping), then increased further to $329 \pm 23 \mu\text{g/l}$ by 12 min after the start of intense pumping, while α -LA did not change significantly from 0800 h. α -LA mRNA expressed as a fold change from 0800 h (time 0). * $P < 0.05$, significantly different from time 0.

epithelium (Berry *et al.* 2001). Kleinberg proposed that GH induces IGF1 from the stroma, which then acts through specific epithelial cell receptors (Kleinberg *et al.* 1990). In the single subject studied with the microarray, GHR and IGF1 were not detectable, while IGF1R mRNA was expressed (Fig. 1). RT-PCR on these genes confirmed the array results (Table 1). Thus, our data are consistent with Kleinberg's proposal and we believe that the RNA isolated from MFG is solely produced by mammary epithelial cells and reflects the cytoplasmic environment of these cells.

The comparative C_t ($\Delta\Delta C_t$) method for relative quantitation (Livak & Schmittgen 2001, Pfaffl 2001) has been used for gene expression studies (Schmittgen & Zakrajsek 2000). While eliminating the need for standard curves, a validated internal control gene is a pre-requisite for its use (Wong & Medrano 2005). However, endogenous genes thought to be stable (e.g. GAPDH, β -actin) have been shown to vary and not be appropriate for normalization purposes (Schmittgen & Zakrajsek 2000, Hamalainen *et al.* 2001, Dheda *et al.* 2004).

Despite utilizing a constant amount of total MFG RNA for each QRT-PCR, R18S, a commonly used endogenous reference gene, varied greater than fivefold from the baseline. Normally used housekeeping genes can vary as much as 34-fold (Dheda *et al.* 2004). Even with the low-density human endogenous control array containing probes for 16 commonly used reference genes, we did not identify a suitable control gene. Interestingly, in our study, the expression pattern of the above 16 genes was similar at each time point (Fig. 2). From these results, one might surmise that the expression of these genes may have a time-dependent expression, or that the breast pumping stimulus induced an upregulation of these genes. If this were the case, then other non-milk-related genes may be necessary for milk synthesis.

We then added an external standard (foreign RNA) to MFG RNA prior to RT-PCR. Others have added cloned artificial RNA molecules or an *in vitro* transcribed RNA (cRNA) without homology to the total RNA as external standards (Huggett *et al.* 2005, Gilsbach *et al.* 2006). However, these procedures are more costly and time-consuming. In our studies, Mm β -actin primers did not cross-react with human DNA/RNA sequences (data not shown), minimizing the potential of cross-contamination with the human samples. The amplification efficiencies for Mm β -actin and α -LA were comparable, and a constant amount of Mm RNA spiked into MFG RNA resulted in a small change in Mm β -actin expression (Fig. 3). Although ideally unchanged, the variability may have been due to methodological errors or the presence of inhibitors in the sample. The $\Delta\Delta C_t$ method of relative quantitation could then be used to quantitate α -LA expression.

In cell culture studies, the expression of α -LA gene has been shown to be hormonally regulated. Addition of PRL, in particular, results in an increase in α -LA mRNA expression in the following 4–6 h (Nagamatsu & Oka 1983, Golden & Rillema 1995). The suckling stimulus evokes a neuroendocrine reflex resulting in PRL secretion (Freeman *et al.* 2000). Simultaneous electric breast pumping elicits a similar PRL response as normal breastfeeding (Zinaman *et al.* 1992). Our two protocols provided similar stimuli (number and length of each pumping episode during the study period) and the similar values obtained for PRL AUC support this.

With the 0800 h intense pumping, an almost 4-fold increase in plasma PRL occurred by 14 min after the start of pumping and α -LA mRNA expression increased significantly 3.5-fold 6 h later, in keeping with the results from *in vitro* studies (Fig. 4). In protocol B, we did not observe a sharp PRL increase during the q30 min pumping; instead, it gradually increased from 0800 to 1200 h, such that with intense pumping from 1200 to 1214 h, the PRL increased by only 50% from the start of intense pumping when compared with the fourfold increase in protocol A. There was no significant change in α -LA mRNA

expression when compared with the baseline sample in protocol B.

Incomplete emptying of the breast at each 2-min pumping in protocol B or a lack of a clear stimulus (large PRL increase) may have hindered further milk synthesis resulting in the low milk yield at 1200 h. Alternatively, milk collection during the q30 min pumping from 0800 to 1200 h, which yielded 132 ± 23 ml, may have emptied the breasts just before intense pumping since the total collected volume was similar to the 0400 h breastfeeding (127 ± 14 ml). Despite a higher peak PRL concentration in protocol B, this was reached gradually rather than acutely as in protocol A. It is possible that the magnitude of PRL change regulates the change in α -LA mRNA expression. On the other hand, breast fullness, the time of day PRL peaks, and the influence of other hormonal factors may have also contributed to the difference in α -LA mRNA expression profiles.

This is the first report to show that high-quality RNA can be isolated from MFG and that mRNA transcripts can be identified and quantitated accurately and reproducibly in humans. We demonstrated that a fourfold increase in PRL concentration was associated with a significant increase in α -LA mRNA expression 6 h following intense pumping at 0800 h; but not when the intense pumping was at noon, preceded by short pulses of breast pumping q30 min. Because of the complexity of milk synthesis, we believe that this research methodology will have great value in unraveling the molecular regulation of milk production and will enable us to further explore the hormonal regulation of gene expression in the lactating mammary gland. The exact mechanism by which PRL regulates α -LA mRNA expression and that of other milk genes requires further investigation.

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