Funct Integr Genomics
DOI 10.1007/s10142-008-0074-y

ORIGINAL PAPER

Onset of lactation in the bovine mammary gland: gene expression profiling indicates a strong inhibition of gene expression in cell proliferation

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Received: 7 November 2007 / Revised: 20 December 2007 / Accepted: 29 December 2007

Abstract The mammary gland undergoes dramatic functional and metabolic changes during the transition from late pregnancy to lactation. To better understand the molecular events underlying these changes, we analyzed expression profiles of approximately 23,000 gene transcripts in bovine mammary tissue about day 5 before parturition and day 10 after parturition. At the cutoff criteria of the signed fold change ≥2 or ≤−2 and false discovery rate (FDR) ≤0.1, a total of 389 transcripts (1.6%) were significantly differentially expressed at the two stages. Of these transcripts with significant changes, 105 were up-regulated while 284 were down-regulated. Gene ontology analysis showed that the main up-regulated genes were those associated with transport activity (amino acid, glucose, and ion transporters), lipid and carbohydrate metabolism (lipoprotein lipase, acetyl-Coenzyme A synthetases, 6-phosphofructo-2-kinase, etc.), and cell signaling factors (protein p8, Rab18, etc.). The main down-regulated genes were associated with cell cycle and proliferation (cyclins, cell division cycle associated proteins, etc.), DNA replication and chromosome organization (centromere proteins, minichromosome maintenance proteins, histone, etc.), microtubule-based processes (microtubule associated protein tau, kinesin, tubulins, etc.), and protein and RNA degradation (proteasome, proteasome activator, RNA binding motif protein, etc.). The increased expression of glucose transporter GLUT1 mRNA during lactation was verified by quantitative reverse transcription/polymerase chain reactin (PCR) ($P < 0.05$). GLUT1 protein also increased twofold during lactation ($P < 0.05$). Furthermore, GLUT1 protein was primarily localized in mammary ductal epithelia and blood vessel endothelia before parturition, but was predominantly localized in the basolateral and apical membranes of mammary alveolar epithelial cells during lactation. Our microarray data provide insight into the molecular events in the mammary gland at the onset of lactation, indicating the up-regulation of genes involved in milk synthesis concomitant with the inhibition of those related to cell proliferation.

Keywords Functional genomics · Gene expression profile · Microarray · Periparturition · Mammary gland · Lactation · Bovine

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Introduction

During pregnancy, systemic endocrine signals promote mammary development and functional differentiation to prepare the mammary gland for milk production at the time of parturition. The rising estrogen levels in blood circulation during early stage of pregnancy in non-lactating cows stimulate mammary ductal morphogenesis and the combined

action of estrogen, progesterone, and prolactin induce the proliferative phase of alveolar morphogenesis (Hovey et al. 2002; Neville et al. 2002; Tucker 2000). The mammary epithelial tissue expansion continues into early lactation (Anderson et al. 1981) and is important because the number of secretory mammary epithelial cells ultimately determines the lactation potential of the animal.

During transition period, from 3 weeks before to 3 weeks after parturition in dairy cow, the mammary gland undergoes dramatic functional and metabolic changes for lactogenesis. Lactogenesis, the initiation of milk synthesis and secretion, includes two stages (Neville et al. 2002). Stage I begins a few weeks before parturition and is characterized by mammary differentiation and progressive expression of milk proteins (caseins, lactalbumins, etc.) as well as secretion of pre-colostrum. Stage II is initiated around parturition and extends for several days after parturition. This stage is characterized by closure of tight junctions between alveolar cells and formation and secretion of colostrums and milk.

The predominant functional and metabolic changes of the mammary gland occur in stage II of lactogenesis. During this stage, the metabolic and nutrient transport activities of mammary epithelial cells increase dramatically from the very limited secretory activity in the non-lactating period to support high levels of milk production. For example, mammary uptake of glucose, the major precursor of lactose, increases ninefold on the day after parturition from day 7 to day 9 prepartum and fivefold from day 2 prepartum in the goat (Davis et al. 1979). This rapid increase of enzymatic and transport activities cannot be accounted for by the increase in epithelial cell numbers in this short period of time and is most likely resulted from increased activities per cell.

The molecular events in the mammary gland underlying lactogenesis are poorly understood. In this study, we attempt to provide some mechanistic insights into the stage II of lactogenesis by profiling gene expression changes from day 5 before parturition to day 10 after parturition in Holstein cows.

Materials and methods

Animals and tissue collection

Bovine mammary tissue samples were obtained from three multiparous Holstein dairy cows at the Miller Research Farm of the University of Vermont under the approval of the University Institutional Animal Care and Use Committee. Each cow underwent mammary biopsy at day 5 (±5) before parturition (d −5) and day 10 (±5) after parturition (d +10) from the right rear quarter and the left rear quarter, respectively. Biopsies were carried out as described by Farr et al. (Farr et al. 1996) and approximately 2g of tissue were obtained in each biopsy. For immunofluorescent staining, small pieces of tissue were fixed on ice in 4% (wt/vol) paraformaldehyde for 4h, rinsed in PBS, and immersed in 0.5M sucrose overnight at 4°C. Tissue blocks were then preserved in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA), rapidly frozen in liquid nitrogen-chilled 2-methylbutane and stored at −80°C. Remaining tissue was immediately frozen in liquid nitrogen and stored at −80°C.

RNA isolation

Total RNA from animal tissue samples was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), purified using QIAGEN RNeasy mini-columns and treated with DNase I (QIAGEN, Valencia, CA, USA). The RNA quantity was assessed by measurement of the optical density at 260/280 nm using a Nanodrop ND-1000 (NanoDrop Technology, Wilmington, DE) and the RNA quality was assessed with the Agilent 2100 Bioanalyzer system using the RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA, USA) and by inspection of 18S and 28S rRNA bands after gel electrophoresis.

Microarray analysis

The microarray analysis (probe labeling, hybridization, and scanning) was performed using Affymetrix GeneChip Bovine Genome Array (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instruction at the University of Vermont Microarray Core Facility. Briefly, 5 μg of total RNA from each tissue sample were first reverse transcribed to the single-stranded cDNAs using a T7 promoter-oligo(dT) primer. The double-stranded cDNAs were then synthesized using T4 DNA polymerase and used as templates for an in vitro transcription to produce the biotinylated cRNAs in the presence of T7 RNA polymerase. The full-length biotinylated cRNAs were fragmented into 35- to 200-base fragments and then hybridized to GeneChip Bovine Genome Arrays for 16h at 45°C in a rotating Affymetrix GeneChip Hybridization Oven 320. After hybridization, arrays were washed and stained with streptavidin–phycocrythrin on an automated Affymetrix GeneChip Fluidic Station F450 station. The arrays were scanned with an Affymetrix GeneChip Scanner 2700 and the images quantified using Affymetrix GeneChip Operating Software (GCOS).

Microarray data analysis

The raw data from three animals before (Dry) and after (Milk) parturition were background-corrected and normalized

probe-level intensities using BioConductor (http://www.bioconductor.org) tools (Gautier et al. 2004). The normalization was performed using the Qspline method of Workman et al. (Workman et al. 2002). An alternative normalization method based on housekeeping genes did not significantly change the results. Expression statistics, E_{ij}^{k}, were calculated for each probe set (i), cow (j), and treatment level ($k \in$ {Milk, Dry}) using the Robust Multichip Average (RMA) method of Speed and coworkers (Bolstad et al. 2003; Irizarry et al. 2003). For each probe set, four statistics were calculated, each reflecting a differential expression in "Milk" samples relative to "Dry", 1) $\Delta E_i = \frac{1}{3}\sum_j \left(RMA_{ij}^{\text{Milk}} - RMA_{ij}^{\text{Dry}}\right)$; 2) the signed fold change, $\text{SFC}_i = \begin{cases} 2^{\Delta E_i}, & \Delta E_i \geq 0 \\ -2^{\Delta E_i}, & \Delta E_i < 0 \end{cases}$; 3) the probability of obtaining $|\Delta E| \geq |\Delta E_i|$ under the null hypothesis (no differential expression) using a paired t test (that is, assuming uncorrelated and normally distributed error with constant variance); 4) the false discovery rate (FDR), using the method of Storey and coworkers (Benjamini et al. 2001; Storey and Tibshirani 2003; Storey et al. 2005).

The selected genes with significant differential expression in two time periods were further analyzed in the context of gene ontology (GO) biological process (www.geneontology.org) (The Gene Ontology (GO) project in 2006) and KEGG biological pathway (Kanehisa et al. 2006) using the GeneSifter online tools (http://genesifter.net/web) and DAVID Bioinformatics Resources 2007 (http://david.abcc.ncifcrf.gov/). The up- and down-regulated genes were extracted from their corresponding GO categories.

Quantitative real-time PCR

Total RNA (1μg) was reverse-transcribed with 0.5μg of Oligo $(dT)_{12–18}$ primer (Invitrogen) and MMLV-Reverse Transcriptase (Invitrogen). The quantitative real time PCR was carried out using SYBR Green Jumpstart (Sigma-Aldrich, St. Louis, MO) and an ABI 7700 (Taqman) instrument (Applied Biosystems, Foster City, CA, USA) as previously described (Zhao et al. 2004). Data are reported as values normalized to the housekeeping gene β-actin. The statistical significance of relative differences was analyzed via Student's paired t test using Minitab 14.20 (http://www.minitab.com).

Western blot analysis

Mammary tissue (1g) were ground to a fine powder using a liquid N_2-chilled mortar and pestle and transferred to a pre-chilled beaker containing five volumes of ice-cold homogenization buffer [0.25M sucrose, 10mM Hepes–NaOH (pH 7.4), 1mM $MgCl_2$, 0.5mM phenylmethylsulfonyl fluoride (PMSF), 1μg/ml pepstatin A, 1μg/ml leupeptin, 1μg/ml chymostatin, 1μg/ml antipain, and 1μg/ml aprotinin]. The suspension was homogenized with a Polytron PT-20 homogenizer at half-maximum speed for two 30-s bursts. All centrifugations were performed at 4˚C. The homogenate was centrifuged for 15min at 1,000g. The supernatant was recovered by decantation and centrifuged for 20min at 17,000g. The resulting supernatant was again centrifuged for 75min at 106,000g. The pellet was suspended in buffer [0.25M sucrose, 10mM Hepes–NaOH (pH 7.4), 1mM $MgCl_2$] and frozen in liquid N_2.

The crude membrane fractions (25μg) were denatured at 95˚C for 4min and resolved on a 12% sodium dodecyl sulfate (SDS)-polyacylamide gel using a Bio-Rad Mini-protein III Electrophoresis Cell (Bio-Rad, Hercules, CA, USA). The proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond ECL, Amersham, Piscataway, NJ, USA). The membrane was incubated overnight at 4°C in Tris-buffered saline (TBS, 20mM Tris, pH 7.4, 137mM NaCl) containing 5% (wt/vol) non-fat milk powder (Bio-Rad) to block non-specific binding. The membrane was washed twice in TBS and then incubated at room temperature for 1h in TBS containing 1% (wt/vol) non-fat milk powder and a 1:100 dilution of a rabbit anti-GLUT1 polyclonal antibody (Chemicon, Temecula, CA, USA). The membrane was rinsed three times in TBS and incubated for 1 h at room temperature in TBS containing 1% (wt/vol) non-fat milk powder and 1:2,000 dilution of a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen). The membrane was finally washed five times in TBS and the immune complex was detected using the West-Pico chemiluminescent kit (Pierce, Rockford, IL, USA). The resulting image was quantified by a Kodak Digital Science Scanner with Kodak Digital Science 1D image analysis software (Kodak, Rochester, NY, USA). The statistical significance of relative differences was analyzed via Student's paired t test using Minitab 14.20.

Immunofluorescence staining

The fixed and OCT-embedded mammary tissue sections described above were sectioned and thaw-mounted on the surface of gelatin-coated slides. The sections were pre-incubated in PBS with 10% (vol/vol) normal donkey serum for 1h at room temperature and then incubated overnight at 4°C in PBS with 1% BSA (wt/vol) containing either 1:100 dilution of a goat polyclonal anti-GLUT1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or normal goat IgG. The sections were washed twice in PBS and incubated in the dark with an AlexaFluor® 568-conjugated Donkey anti-Goat IgG secondary antibody (Molecular Probes, Eugene, OR, USA) at 1:400 dilution for 1h at room temperature. After washing twice in PBS, tissue sections were counterstained with SYTOX (Molecular Probes) with

1:10,000 dilution in PBS. The sections were finally washed, mounted onto slides with Aqua Poly/Mount (PolySciences, Warrington, PA, USA), and examined under the Zeiss LSM 410 Confocal Microscopy (Carl Zeiss Optical, Chester, VA, USA).

Results

Microarray analysis

The Affymetrix GeneChip Bovine Genome Arrays, which contain 24,072 probe sets representing more than 23,000 transcripts and 19,000 UniGene clusters, were used to profile gene expression changes in the mammary gland of Holstein cows about 5days before parturition and 10days after parturition. At the cutoff criteria of the signed fold change ≥ 2 or ≤ -2 and FDR ≤ 0.1, a total of 389 transcripts (1.6% of total probe sets on the array) were significantly differentially expressed at the two stages. Of these transcripts with significant changes, 105 (0.4%) were up-regulated while 284 (1.2%) were down-regulated. Table 1 lists the top 25 up-regulated and top 25 down-regulated transcripts. Lactoperoxidase and parathyroid hormone-like hormone topped the up-regulated gene list and increased about 80- and 30-fold, respectively, from late pregnancy to early lactation. The other top up-regulated genes included myocin-binding protein C, lipoprotein lipase, spermadhesin 1 (an acidic seminal fluid protein precursor), 5′-nucleotidase (ecto), nitric oxide synthase trafficker, protein p8, long-chain acyl-CoA synthetase homolog 1, mono(ADP-ribosyl)transferase, lipin 1, pim-1 oncogene, and several transporter proteins. Topping the down-regulated gene list were two hypothetical proteins with unknown functions. Other major down-regulated genes included nucleolar and spindle-associated protein 1, centromeric proteins, topoisomerase II, aquaporin 5, ribonucleoside-diphosphate reductase M2 chain, cyclin B1, G protein-coupled receptor 126, Stathmin 1, and serine/threonine-protein kinases.

Gene ontology (GO) analysis

The selected genes with significant modification in their expression at the two different stages were analyzed in the context of GO biological process. The GO analysis dynamically assigns different genes to the different GO biological-process categories and provides a z score for each category to represent a statistical measure of the relative amounts of gene expression changes in the given GO term. This approach revealed that the major changes of gene expression at the two stages were associated with the cellular (~40%) and physiological (~37) processes in biological process ontology categories (Fig. 1a) and with binding activities (~48%) and catalytic activities (~28%) in molecular function ontology categories (Fig. 1b).

The GO analysis revealed that the major non-redundant GO biological processes that were significantly associated with up-regulated genes in early lactation are metabolic process, transport process, and cell signal transduction (Table 2). More than 18 up-regulated genes (21 of 105 transcripts) were associated with the lipid, carbohydrate, protein, and nucleic acid metabolic processes (mainly anabolic metabolism). The genes involved in lipid metabolism included lipoprotein lipase, long-chain acyl-CoA synthetase homolog 1, acetyl-CoA synthetase 2 (AMP forming)-like and aldo-keto reductase family 1. Involved in carbohydrate metabolism were 6-phosphofructo-2-kinase, pyruvate dehydrogenase kinase 4, and carbonic anhydrase VI. The increased expression of anhydrase VI during lactation is different from the significant decrease of anhydrase III expression in mouse mammary gland (Lemkin et al. 2000), indicating different roles of these two enzymes or species differences. The 5' nucleotidase ecto, mono-ADP-ribosyltransferase 3, pim-1 oncogene, phosphoserine aminotransferase 1, retinol dehydrogenase 11, UDP-glucuronosyltransferase 2B4 precursor, serine (or cysteine) proteinase inhibitor 2, and *n*-acylglucosamine 2-epimerase were associated with protein and nucleic acid metabolism. More than 10 genes (11 transcripts) were linked to transport processes of amino acids (SLC7A5, SLC1A4, SLC1A5 and SLC6A9), glucose (SLC2A1), ions (SLC39A12, SLC34A2 and SLCO1A2), and other molecules (SLC25A21 and VLDL-R2). In addition, at least five genes (six transcripts) were cell signal transduction molecules, including protein p8, Rab-18, SH3 domain-binding protein 5, kruppel-like factor 15 and ankyrin repeat and SOCS box-containing 11.

The GO analysis linked more than 61 significantly down-regulated genes (68 of 284 transcripts) to cell division, cell cycle, and other related biological processes including DNA replication, chromosome organization, and biogenesis and mictotubule-based process (Table 3). These genes included cyclins B1, B2, D1, and E2, cell division-associated proteins cks2, 7, 8, 3, 20 homolog and 5, mitotic checkpoint proteins BUB1 beta, Nek2, CHK1 homolog, *c*-Myc proto-oncogene and Madp2 homolog, nucleolar and spindle-associated protein 1, centromere proteins F, A, and N, topoisomerase II alpha, minichromosome maintenance proteins and homologs, core histone macro-H2A.2, microtubule-associated protein tau, kinesin-like proteins, and tubulin beta 5. In addition, among other down-regulated genes, nine (10 transcripts) including proteasome proteins and lysozyme C were linked to proteolysis and protein catabolic process; four (laminin alpha1 precusor, collagen XXII alpha 1, hyaluronan-mediated motility receptor and lysyl oxidase-like 4) were involved in extracellular matrix formation and cell adhesion; and five

Table 1 Top 50 of up-regulated or down-regulated transcripts in the bovine mammary gland at the early lactation compared to the late pregnancy

Affymetrix probe identifier	GenBank accession	Gene description	SFC	*P* value	FDR
Up-regulated					
bt.4784.1.s1_at	NM_173933	Lactoperoxidase	79.96	0.000	0.059
bt.12848.1.s1_at	NM_174753	Parathyroid hormone-like hormone (PTHLH)	30.41	0.005	0.088
bt.25544.1.a1_at	CK770973	Transcribed locus	18.43	0.020	0.099
bt.23229.1.s1_at	BE758247	Similar to myosin binding protein C, slow type	15.90	0.003	0.087
bt.5387.1.s1_at	BG688620	Lipoprotein lipase	10.97	0.006	0.089
bt.19232.1.a1_at	CB460236	Similar to CG6424-PA, isoform A	9.79	0.020	0.099
bt.27735.1.a1_at	CK848929	Glycosylation-dependent cell adhesion molecule 1	9.29	0.017	0.096
bt.2881.1.s1_at	CK950053	Similar to *N*-myc downstream regulated gene 4	8.72	0.019	0.098
bt.2849.1.s1_at	CK974512	Similar to Pan troglodytes solute carrier family 7, (cationic amino acid transporter, y + system) member 11	7.91	0.000	0.070
bt.457.1.s1_at	NM_174616	Spermadhesin 1	7.34	0.007	0.089
bt.17665.1.a1_at	CB452221	Solute carrier family 25 (mitochondrial oxodicarboxylate carrier), member 21	6.72	0.000	0.059
bt.5515.1.s1_at	NM_174129	5' nucleotidase, ecto (cd73)	6.45	0.009	0.089
bt.17865.1.a1_at	CK981854	Nitric oxide synthase trafficker	6.37	0.005	0.088
bt.20162.1.s1_at	BE683886	Transcribed locus	5.72	0.019	0.098
bt.10310.1.s1_at	CB169112	Similar to myosin binding protein C, slow type	5.37	0.006	0.089
bt.23597.1.s1_at	CK848830	Similar to Nuclear protein 1 (Protein p8)	5.31	0.002	0.082
bt.5528.1.s1_at	NM_174613	Solute carrier family 7 (cationic amino acid transporter, y + system), member 5	4.99	0.008	0.089
bt.26381.1.a1_at	CK966630	Transcribed locus	4.97	0.000	0.059
bt.26921.1.a1_at	CK772265	Similar to very long-chain acyl-CoA synthetase homolog 1; VLCS-H1	4.59	0.000	0.064
bt.2367.1.a1_at	NM_174129	5′-nucleotidase, ecto (cd73)	4.45	0.005	0.088
bt.29697.1.s1_at	XM_593306	mono (ADP-ribosyl)transferase	4.34	0.001	0.076
bt.6642.1.s1_a_at	CB424184	Similar to lipin 1	4.33	0.009	0.089
bt.16611.1.a1_at	CK778694	Similar to midline 1 isoform alpha	4.28	0.004	0.088
bt.6800.1.a1_at	BF440269	Similar to RasGEF domain family, member 1B	4.04	0.001	0.076
bt.272.1.s1_at	NM_174144	pim-1 oncogene	3.96	0.001	0.076
Down-regulated					
bt.27743.2.a1_at	CK849080	Similar to thrombospondin type 1 domain containing	−29.50	0.021	0.100
bt.18776.1.s1_at	CK979795	KIAA0101 protein	−16.17	0.004	0.088
bt.25412.1.a1_at	BE723538	Nucleolar and spindle associated protein 1	−12.62	0.002	0.082
bt.16453.1.a1_at	CK849813	Transcribed locus	−12.09	0.011	0.091
bt.28305.1.s1_at	CK946096	Similar to hepatocellular carcinoma antigen gene 520	−10.99	0.012	0.092
bt.12328.1.s1_at	BF073044	Similar to centromere protein F (350/400kD)	−10.13	0.004	0.088
bt.20277.1.s1_at	CB443446	Similar to topoisomerase II	−10.00	0.002	0.083
bt.21523.1.s1_at	BE663592	Similar to Histone H3-like centromeric protein A (Centromere protein A) (CENP-A)	−9.93	0.002	0.083
bt.1296.1.s1_at	BI537914	Transcribed locus	−9.39	0.007	0.089
bt.13573.1.a1_at	BP110236	Similar to Homo sapiens CDC28 protein kinase regulatory subunit 2	−9.05	0.005	0.089
bt.28379.1.s1_at	CK951457	Similar to ubiquitin-like, containing PHD and RING finger domains, 1	−8.53	0.005	0.089
bt.28366.1.a1_at	CK979039	Strongly similar to epithelial cell transforming sequence 2 oncogene protein	−8.19	0.000	0.059
bt.15980.1.a1_at	CB443402	Cyclin B1	−8.15	0.002	0.082
bt.25661.1.a1_at	CK772666	Similar to Mitotic checkpoint serine/threonine-protein kinase BUB1 beta	−8.10	0.006	0.089
bt.16712.1.a1_at	CB454267	Similar to aquaporin 5	−7.08	0.006	0.089
bt.5934.1.s1_at	CB172104	Strongly similar to Serine/threonine-protein kinase Nek2	−7.03	0.003	0.088
bt.1296.2.s1_at	BE666806	Transcribed locus	−6.91	0.003	0.087
bt.8633.1.a1_at	BI535494	Similar to PRR11 protein	−6.76	0.004	0.088
bt.10648.1.s1_at	CK979761	Similar to Ribonucleoside-diphosphate reductase M2 chain (Ribonucleotide reductase small chain)	−6.65	0.004	0.088
bt.10340.1.s1_at	BM030692	Similar to G protein-coupled receptor 126	−6.35	0.010	0.091
bt.17179.1.s1_at	CK970204	Transcribed locus	−6.34	0.004	0.088

Table 1 (continued)

Affymetrix probe identifier	GenBank accession	Gene description	SFC	*P* value	FDR
bt.10106.1.s1_at	CB167909	Similar to tripartite motif-containing 9 (TRIM9)	−6.25	0.005	0.089
bt.28185.1.s1_at	CK962142	Similar to Rac GTPase activating protein 1, transcript variant 4	−6.09	0.003	0.083
bt.3137.1.s1_at	NM_001034790	Stathmin 1/oncoprotein 18	−6.00	0.008	0.089
bt.10007.1.a1_at	CK944937	Similar to cytoskeleton associated protein 2, transcript variant 3 (CKAP2)	−5.83	0.002	0.082

SFC signed fold change, *FDR* false discovery rate

(seven transcripts) encodes calcium binding proteins (centrin 4, delta-like homolog, S100 calcium-binding protein A10, calmodulin-like 4, and neurocalcin delta). Four G-protein-signaling molecules (G-protein-coupled receptor 126 and 37 precursor, G-protein signaling modulator 2, and regulator of G-protein signaling 2) were also significantly down-regulated.

Changes of milk protein gene expression

None of the milk protein genes (caseins, alpha-lactalbumin, and beta-lactoglobulin) showed significant changes in expression from late pregnancy to early lactation based on our set criteria. The expression changes of these genes are listed in Table 4. Although expression of all milk protein genes showed a signed fold change from 1.20 to 1.68, the false discovery rate of all of these genes is larger than the cutoff rate of 0.1 (10%), thus deemed to be insignificant.

Verification of glucose transporter mRNA expression by quantitative RT-PCR and analysis of their protein expression and localization changes

Glucose uptake in the mammary gland plays a critical role in milk synthesis because glucose is the major substrate of lactose synthesis. Through osmosis, lactose largely controls milk volume (Holt 1983). Thus, expression of the major glucose transporters in the bovine mammary gland, GLUT1 and GLUT8 (Zhao et al. 1993, 1996, 2004; Zhao and Keating 2007), were verified by quantitative real time PCR (qPCR). As shown in Fig. 2, the expression change of GLUT1 mRNA around parturition obtained by qPCR matched the data by microarray. Expression changes of GLUT8 obtained by both methods were not statistically significant. Western blot analysis and immunofluorescent staining of GLUT1 were further carried out to examine the changes of GLUT1 protein abundance and localization in

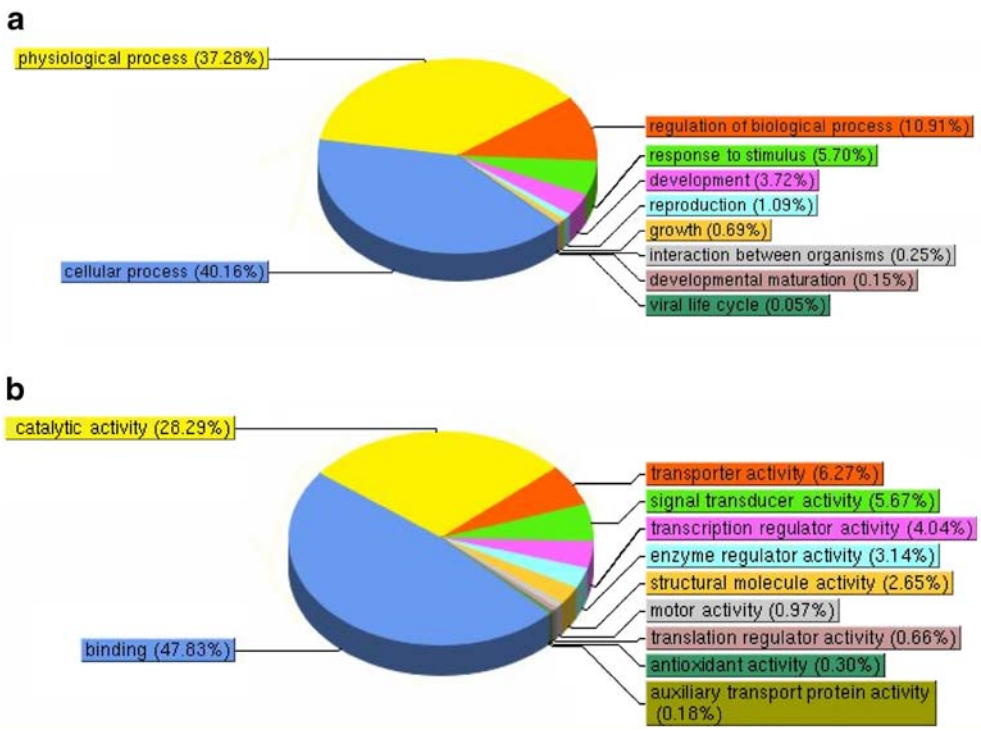

Fig. 1 Pie charts of the major biological process (**a**) and molecular function (**b**) ontology categories associated with the significantly differentially expressed genes in the bovine mammary gland at early lactation compared to late pregnancy. Ontology was determined and the charts were generated by the ontology function of GeneSifter (http://www.genesifter.net/web/)

Table 2 List of GO biological processes associated with significantly up-regulated genes in the bovine mammary gland at the early lactation compared to the late pregnancy

Affymetrix probe identifier	GenBank accession	Gene description	SFC	*P* value	*FDR*
Metabolic process					
bt.5387.1.s1_at	BG688620	Lipoprotein lipase	10.97	0.006	0.089
bt.5515.1.s1_at	NM_174129	5′ nucleotidase, ecto	6.45	0.001	0.039
bt.26921.1.a1_at	CK772265	Similar to very long-chain acyl-CoA synthetase homolog 1; VLCS-H1	4.59	0.000	0.064
bt.29697.1.s1_at	BM256348	Mono-ADP-ribosyltransferase 3 (ART3)	4.34	0.000	0.034
bt.272.1.s1_at	NM_174144	pim-1 oncogene	3.96	0.001	0.076
bt.12314.1.s1_at	BI774743	6-phosphofructo-2-kinase (PFKFB1)	3.76	0.047	0.116
bt.23094.1.a1_at	D88749	Aldo-keto reductase family 1, member C1 (20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	3.47	0.047	0.116
bt.3372.2.s1_a_at	XM_588987	Similar to putative lysophosphatidic acid acyltransferase	3.91	0.009	0.089
bt.3372.3.s1_a_at	XM_866018	isoforms	3.16	0.007	0.089
bt.13588.3.a1_at	XM_612314	Similar to phosphoserine aminotransferase isoform 1	3.15	0.020	0.099
bt.17242.1.a1_at	CK954048	Cytochrome P450 subfamily 2B	2.97	0.011	0.091
bt.18951.1.s1_at	XM_582373	Similar to retinol dehydrogenase 11 (retinal reductase 1)	2.42	0.002	0.082
bt.5630.1.s1_at	XM_865628	(RalR1)	2.39	0.004	0.088
bt.18951.2.s1_at	CB452122		2.09	0.016	0.082
bt.19825.1.s1_at	XM_587609 XM_864676	Similar to UDP-glucuronosyltransferase 2B4 precursor (UDPGT) (Hyodeoxycholic acid) (HLUG25) (UDPGTh-1)	2.30	0.015	0.095
bt.24210.1.s1_at	BP101056	Similar to acyl-CoA synthetase long-chain family member 1	2.26	0.002	0.083
bt.15996.1.s2_at	CK849030	Acetyl-Coenzyme A synthetase 2 (AMP forming)-like	2.21	0.019	0.098
bt.23505.1.s1_at	BE753959	Pyruvate dehydrogenase kinase, isozyme 4	2.15	0.006	0.089
bt.13073.1.s1_at	XM_582291	Serine (or cysteine) proteinase inhibitor, clade b, member 2	2.15	0.013	0.093
bt.2629.1.s1_at	XM_594363	Similar to *n*-acylglucosamine 2-epimerase (glcnac 2-epimerase) (*n*-acetyl-*d*-glucosamine 2-epimerase) (age)	2.11	0.001	0.076
bt.47.1.s1_at	NM_173898	Carbonic anhydrase VI	2.00	0.008	0.089
Transport process					
bt.26950.1.a1_at	CK778659	Similar to solute carrier family 39 (zinc transporter), member 12 (SLC39A12)	11.70	0.023	0.100
bt.17665.1.a1_at	NM_001015587	Solute carrier family 25 (mitochondrial oxodicarboxylate carrier), member 21 (SLC25A21)	6.72	0.000	0.059
bt.5528.1.s1_at	NM_174613	Solute carrier family 7 (cationic amino acid transporter, y + system), member 5 (SLC7A5)	4.99	0.008	0.089
bt.5484.1.s1_at	NM_174489	Very low density lipoprotein receptor, VLDL-R2	3.58	0.008	0.089
bt.28214.1.s1_at	CF768959	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4 (SLC1A4)	3.19	0.003	0.088
bt.13712.1.s1_at			2.31	0.005	0.089
bt.84.1.s1_at	NM_174661	Solute carrier family 34 (sodium phosphate), member 2 (SLC34A2)	3.14	0.005	0.088
bt.5548.1.s1_at	NM_174601	Solute carrier family 1 (neutral amino acid transporter), member 5 (SLC1A5)	2.83	0.001	0.076
bt.8946.2.s1_a_at	AY052775	Organic anion transporting polypeptide 1a2 (SLCO1A2)	2.29	0.018	0.098
bt.4855.1.s1_a_at	NM_174612	Solute carrier family 6 (neurotransmitter transporter, glycine), member 9 (SLC6A9)	2.22	0.007	0.089
bt.4646.1.s1_at	NM_174602	Solute carrier family 2 (facilitated glucose transporter), member 1 (SLC2A1)	2.10	0.015	0.095
Cell signal transduction					
bt.23597.1.s1_at	XM_867457 XM_880232	Similar to Nuclear protein 1 (Protein p8) (Candidate of metastasis 1)	5.31	0.002	0.082
bt.20678.1.a1_at	CB459886	Similar to Ras-related protein Rab-18	3.91	0.006	0.089
bt.8619.1.s1_at	BI534841	Similar to SH3 domain-binding protein 5 (SH3 domain-binding protein that preferentially associates with BTK)	2.99	0.000	0.059
bt.20680.1.s1_at	CB426179		2.27	0.005	0.088
bt.6404.1.s1_at	XM_615450	Kruppel-like factor 15	2.43	0.015	0.095
bt.7651.1.s1_at	CK772226	Ankyrin repeat and SOCS box-containing 11	2.17	0.010	0.090

SFC = signed fold change, FDR = false discovery rate

Table 3 List of GO biological processes associated with significantly down-regulated genes in the bovine mammary gland at the early lactation compared to the late pregnancy

Affymetrix probe identifier	GenBank accession	Gene description	SFC	*P* value	*FDR*
Cell division and Cell cycle					
bt.25412.1.a1_at	BE723538	Nucleolar and spindle associated protein 1	−12.62	0.002	0.082
bt.13573.1.a1_at	BP110236	Similarity to cell division control protein cks2	−9.05	0.005	0.089
bt.15980.1.a1_at	CB443402	Cyclin B1	−8.15	0.002	0.082
bt.25661.1.a1_at	CK772666	Similar to Mitotic checkpoint serine/threonine-protein kinase BUB1 beta	−8.10	0.006	0.089
bt.41.1.s1_at	NM_174264	Cyclin B2	−7.04	0.024	0.100
bt.5934.1.s1_at	CB172104	Similar to Serine/threonine-protein kinase Nek2	−7.03	0.003	0.088
bt.10253.1.a1_at	CK971800	Similar to CHK1 checkpoint homolog	−5.58	0.013	0.094
bt.2353.1.s1_at	CK772080	Baculoviral IAP repeat-containing 5 (survivin)	−5.34	0.015	0.095
bt.6441.1.s1_at	CK952615	Cell division cycle associated 7	−4.92	0.021	0.100
bt.27319.1.a1_at	CK848419	Similar to division cycle associated 8	−4.75	0.000	0.069
bt.16538.2.a1_at	AV604909	Cyclin D1	−4.06	0.010	0.091
bt.24862.1.a1_at	CK966382		−3.34	0.013	0.094
bt.11587.3.a1_a_at	CK774460	Similar to Sperm-associated antigen 5 (Astrin) (Mitotic spindle associated protein p126) (MAP126)	−4.02	0.004	0.088
bt.21513.1.a1_at	CK846625	Similar to BUB1 budding uninhibited by benzimidazoles 1 homolog	−3.97	0.009	0.089
bt.29462.1.s1_at	CK972892	Similar to cell division cycle associated 2	−3.90	0.008	0.089
bt.24202.1.s1_at	CK957201	Cell division cycle 2, G1 to S and G2 to M	−3.70	0.009	0.089
bt.10696.1.s1_at	CK846897	Cell division cycle associated 3 (CDCA3)	−3.64	0.008	0.089
bt.19819.1.s1_at	AAR12662 AAR98745	asp (abnormal spindle)-like, microcephaly associated (drosophila)	−3.61	0.014	0.094
bt.21164.1.s1_at	XM_588339 XM_872163	Similar to Myc proto-oncogene protein (c-Myc) (Transcription factor p64)	−3.40	0.009	0.089
bt.2.1.s1_at	NM_174016	Cell division cycle 2, G1 to S and G2 to M	−3.38	0.002	0.083
bt.4995.1.a1_at	CB447825	Similar to anillin, actin binding protein (scraps homolog, Drosophila)	−3.28	0.016	0.096
bt.4995.2.s1_at	CB418450		−3.10	0.005	0.088
bt.12986.1.s1_at	CK977019	Similar to mitotic feedback control protein Madp2 homolog	−3.20	0.008	0.089
bt.986.1.a1_at	AW346089	Similar to F-box only protein 5	−3.17	0.002	0.083
bt.639.1.s1_at	XM_593387	Similar to Cell division cycle protein 20 homolog (p55CDC)	−3.14	0.011	0.092
bt.16141.1.s1_at	CK778261	Cyclin E2	−3.03	0.011	0.091
bt.16047.1.s1_at	CB426310	Cell division cycle associated 5	−2.90	0.014	0.095
bt.26683.2.s1_at	CK978780	Aurora-A	−2.50	0.011	0.091
bt.10391.1.s1_at	XM_615288	Similar to p53-regulated DDA3 isoform b	−2.02	0.001	0.076
DNA replication and Chromosome organization and biogenesis					
bt.12328.1.s1_at	BF073044	Similar to centromere protein F (350/400kD)	−10.13	0.004	0.088
bt.20277.1.s1_at	CB443446	Similar to topoisomerase (DNA) II alpha 170kDa	−10.00	0.002	0.083
bt.21523.1.s1_at	XM_869623	Centromere protein-A	−9.93	0.002	0.083
bt.10648.1.s1_at	CK979761	Similar to Ribonucleoside-diphosphate reductase M2 chain	−6.65	0.004	0.088
bt.21433.1.s1_at	BF776813	MCM6 minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)	−5.49	0.017	0.096
bt.21433.2.s1_at	AV607790		−4.68	0.021	0.100
bt.11711.1.a1_at	CK775474	Similar to high-mobility group box 3	−5.39	0.011	0.091
bt.20262.1.s1_at	CK769686	similar to minichromosome maintenance protein 4 (DNA replication licensing factor (CDC21 homolog)	−4.66	0.012	0.092
bt.11129.1.s1_at	CK849016	Similar to DNA replication licensing factor MCM3	−4.26	0.002	0.082
bt.2882.1.a1_at	AV591121	Similar to centrin 4	−4.21	0.011	0.091
bt.2882.2.s1_at	BM106308		−3.29	0.014	0.095
bt.6129.1.s1_at	CK770134	Similar to kinetochore associated 2	−3.56	0.006	0.089
bt.13336.1.a1_at	CB442617	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)	−3.52	0.006	0.089
bt.13336.2.s1_at	XM_586752		−2.66	0.014	0.095
bt.22976.1.s1_at	XM_863831		−2.58	0.018	0.098

Table 3 (continued)

Affymetrix probe identifier	GenBank accession	Gene description	SFC	*P* value	*FDR*
Bt.3371.1.S1_at	CK947569	Similar to minichromosome maintenance protein 2	−3.23	0.019	0.098
bt.964.1.s1_at	CK847835	Similar to Thymidine kinase, cytosolic	−3.04	0.009	0.090
bt.28797.2.s1_at	CK940769	Centromere protein N	−2.94	0.007	0.089
bt.10931.1.s1_at	CB464823	Similar to Core histone macro-H2A.2 (Histone macroH2A2)	−2.88	0.004	0.088
bt.4198.1.s1_at	XM_583471	Similar to deoxythymidylate kinase (thymidylate kinase)	−2.80	0.006	0.089
bt.6397.2.s1_at	CK977129	High-mobility group box 2	−2.70	0.010	0.091
bt.9140.1.s1_at	BM251387	Geminin-like	−2.70	0.006	0.089
bt.12087.1.a1_at	CK847815	Similar to structural maintenance of chromosomes 2-like 1	−2.66	0.002	0.082
bt.13480.1.s1_at	CB531875	Similar to proliferating cell nuclear antigen	−2.40	0.007	0.089
bt.858.1.s1_at	CB447832	Similar to CTF18, chromosome transmission fidelity factor 18 homolog	−2.39	0.007	0.089
bt.12465.1.a1_at	CK972197	Similar to ribonucleotide reductase M1	−2.35	0.001	0.076
bt.18230.1.s1_a_at	BF651503	Nuclear autoantigenic sperm protein (histone-binding)	−2.31	0.015	0.095
bt.27805.1.s1_at	CK944283	Similar to proliferation associated nuclear element 1	−2.23	0.008	0.089
bt.27302.1.s1_at	XM_587221	Similar to minichromosome maintenance protein 2	−2.19	0.018	0.098
bt.9090.1.s1_at	XM_583077	Similar to chromobox protein homolog 1 Heterochromatin protein 1 homolog beta)	−2.08	0.006	0.089
	XM_871608				
Microtubule-based process					
bt.3137.1.s1_at	CK848121	Stathmin 1/oncoprotein 18	−6.01	0.008	0.089
bt.20455.1.s1_at	BF076898	Microtubule associated protein tau	−5.70	0.001	0.076
bt.467.1.s1_at	NM_174106		−2.99	0.012	0.092
bt.17939.1.s1_at	CB436656	Similar to Lamin-B1	−4.83	0.005	0.088
bt.13476.1.s1_at	CK950633	Similar to Kinesin-like protein KIF23 (Mitotic kinesin-like protein 1) (Kinesin-like protein 5)	−3.96	0.007	0.089
bt.18081.1.a1_at	CB439354	Kinesin family member 20A	−2.88	0.006	0.089
bt.22803.1.s1_at	CK849039	Similar to tubulin, beta 5	−2.76	0.017	0.096
bt.2612.1.s1_at	NM_174620	TCTEL1 protein	−2.62	0.009	0.089
bt.15730.1.s1_at	CK973983	similar to Kinesin-like protein KIF2C	−2.28	0.005	0.089
bt.28607.1.s1_at	CB165581	Similar to cytoplasmic dynein light chain 1	−2.15	0.013	0.094
bt.2307.1.s1_s_at			−2.02	0.21	0.099
Proteolysis and Catabolic process					
bt.27759.1.a1_at	CK849867	Similar to Indoleamine 2,3-dioxygenase (IDO)	−4.77	0.006	0.089
bt.2725.1.s1_at	CK969779	Similar to Ubiquitin-conjugating enzyme E2 C (Ubiquitin-protein ligase C) (UbcH10)	−4.51	0.003	0.087
bt.25855.3.a1_a_at	CK846999	Glutamyl aminopeptidase (aminopeptidase A)	−4.21	0.015	0.095
bt.25855.2.s1_at	BM445541		−3.92	0.013	0.094
bt.209.2.s1_a_at	M26245	Lysozyme C-2 (LYZ2)	−3.54	0.009	0.089
bt.4781.1.s1_at	NM_174496	A disintegrin and metalloproteinase domain 10	−2.86	0.014	0.094
bt.3624.1.s1_at	CK775170	Proteasome (prosome, macropain) subunit, beta type, 10	−2.27	0.003	0.085
bt.15729.1.s1_at	CK849143	Proteasome activator subunit 2	−2.17	0.009	0.089
bt.23098.2.s1_at	CB456375	Similar to tRNA splicing endonuclease 34 homolog	−2.10	0.008	0.089
bt.26700.1.s1_at	CK775261	Histamine *N*-methyltransferase	−2.10	0.004	0.088
Cell adhesion (extracellular matrix)					
bt.26511.1.s1_at	CB170501	Similar to laminin, alpha 1 precursor	−4.14	0.008	0.089
bt.21896.1.s1_at	AW314867	Similar to collagen, type XXII, alpha 1	−4.06	0.013	0.094
bt.1518.1.s1_at	AW430532	Hyaluronan-mediated motility receptor (RHAMM)	−3.00	0.005	0.088
bt.12297.1.s1_at	NM_174384	Lysyl oxidase-like 4	−2.14	0.014	0.094
Calcium ion binding					
bt.2882.1.a1_at	AV591121	Similar to centrin 4	−4.21	0.011	0.091
bt.2882.2.s1_at	BM106308		−3.29	0.014	0.095
bt.5223.1.s1_at	NM_174037	delta-like homolog (Drosophila)	−4.16	0.005	0.089

Table 3 (continued)

Affymetrix probe identifier	GenBank accession	Gene description	SFC	*P* value	*FDR*
bt.5037.1.s2_at	CK966810	S100 calcium-binding protein A10	−3.57	0.021	0.100
bt.5037.1.s1_at	NM_174650		−3.53	0.004	0.088
bt.15294.1.s1_at	CK775501	Similar to calmodulin-like 4	−2.93	0.002	0.082
bt.10096.1.s1_at	CK774508	Neurocalcin delta	−2.78	0.003	0.087
G-protein signaling					
bt.10340.1.s1_at	BM030692	Similar to G protein-coupled receptor 126	−6.35	0.010	0.091
bt.2150.1.s1_at	AW655450	Similar to G-protein signalling modulator 2 (AGS3-like)	−4.06	0.001	0.076
bt.10349.1.s1_at	CK772342	Similar to Probable G-protein coupled receptor 37 precursor	−3.45	0.018	0.098
bt.10855.1.s1_at	CB169117	Similar to regulator of G-protein signalling 2, 24kDa	−2.49	0.013	0.093

SFC signed fold change, *FDR* false discovery rate

these samples. As shown in Fig. 3, a twofold increase in GLUT1 protein was also observed in the mammary gland in early lactation compared to the late pregnant stage ($P < 0.05$). It is worth mentioning that the increase in GLUT1 protein levels appears to correlate well with days relative to parturition, increasing from day −10 to day −1 and further to early lactation. Consistent with the data obtained in Western blot analysis, immunofluorescent staining was also shown a stronger GLUT1 staining in lactating mammary gland and the staining also appeared to become stronger from day −10 to day −1 before parturition (Fig. 4). In addition, GLUT1 appeared to be mainly localized in the basolateral membrane of mammary epithelial cells at day 3 or earlier before parturition and then localized in both basolateral and apical membranes at day 1 before parturition and in lactation. Control staining with normal goat IgG essentially showed no signal (data not shown).

Discussion

In this study, microarray analysis was carried out to profile gene expression changes in the bovine mammary gland at the onset of lactation using the Affymetrix GeneChip Bovine Genome Arrays, which contain 24,072 probe sets representing more than 23,000 transcripts. Unfortunately, many of these bovine transcripts have not been annotated and the majority of the available annotations were based on the sequence similarity to other species. In this study, only the annotations based on the strong sequence similarity were used and these annotations should be considered to be reliable.

Our microarray data indicated that more than twice as many genes were down-regulated than up-regulated in early lactation (284 vs 105 transcripts). This is surprising considering that it is reasonable to expect that lactation requires the increased expression or turning-on of many genes. However, our array analysis showed no significant changes in milk protein mRNA expression as previously observed in the lactating mammary gland compared to the non-lactating mammary gland (Suchyta et al. 2003). It has been previously shown in mouse (Robinson et al. 1995; Rijnkels et al. 1997), rat (Rosen et al. 1975), and rabbit (Shuster et al. 1976) that the expression of milk protein genes starts in early to mid-pregnancy, increases throughout the pregnancy and reaches a plateau in late pregnancy and early lactation. This expression pattern may be similar in the cow. In addition, lactose synthesis has also shown to

Table 4 Expression changes of milk protein genes in the bovine mammary gland in early lactation compared to late pregnancy

Affymetrix probe identifier	GenBank accession	Gene description	SFC	*P* value	FDR
bt.3683.1.s1_at	NM_181029	Casein, alpha-S1 (CSN1S1)	1.38	0.073	0.129
bt.5354.1.s1_at	NM_174528	Casein, alpha-S2 (CSN1S2)	1.38	0.107	0.144
bt.5381.2.s1_x_at	NM_181008	Casein, beta (CSN2)	1.68	0.150	0.161
bt.5381.1.s1_at	M16645		1.27	0.100	0.141
bt.5381.2.a1_at	NM_181008		1.20	0.177	0.173
bt.583.1.s1_a_at	NM_174294	Casein, kappa	1.22	0.031	0.106
bt.5396.1.s1_at	NM_174378	Lactalbumin, alpha	1.51	0.106	0.144
bt.385.1.s1_at	NM_173929	Lactoglobulin, beta	1.24	0.181	0.174

SFC signed fold change, *FDR* false discovery rate

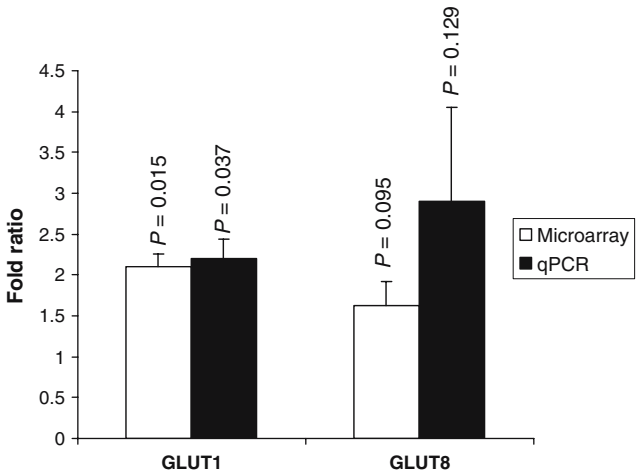

Fig. 2 Comparison of the bovine mammary gland mRNA expression data (fold changes between early lactation and late pregnancy) of glucose transporters GLUT1 and GLUT8 obtained by microarray (open bar) and real-time PCR (*filled bar*). The statistical information in each analysis is indicated by *P* value

start in mid-pregnancy in the rabbit (Mellenberger and Bauman 1974) and cow (Mellenberger et al. 1973). Thus, it is possible that many genes supporting lactation may be already expressed at a high level at the late pregnant stage.

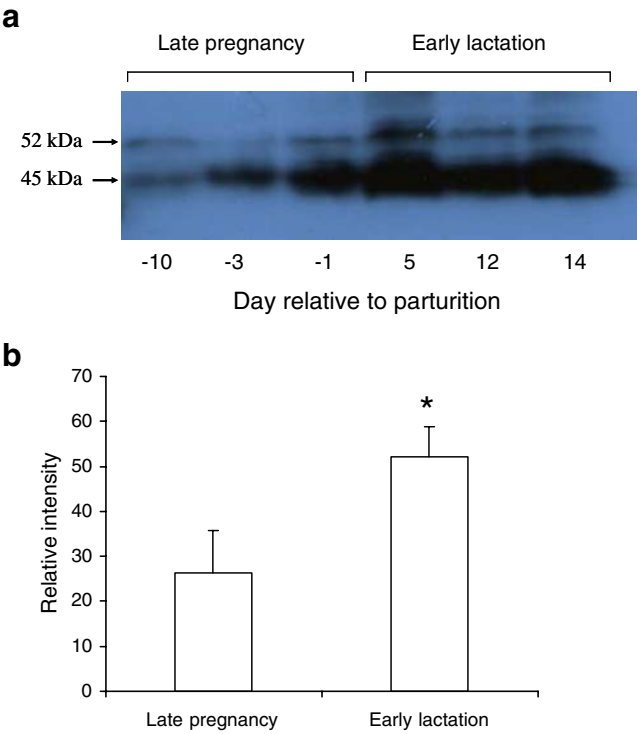

Fig. 3 Western blot analysis of GLUT1 protein abundance in the bovine mammary gland in late pregnancy and early lactation. **a** The gel image of Western blot analysis. The exact biopsy date of each sample is indicated by the day relative to parturition. The sizes in kilodaltons (kDa) of the detected GLUT1 bands are indicated by arrows. **b** Densitometry of the data in (**a**). The *asterisk* indicates significant differences relative to late pregnancy group ($P<0.05$)

The GO analysis still linked many up-regulated genes to metabolism and transport processes, consistent with the observations in mouse mammary gland (Rudolph et al. 2003, 2007). Among these genes, lipoprotein lipase (LPL) is an enzyme that hydrolyzes lipids in lipoproteins in chylomicrons and very low density lipoproteins (VLDL) into fatty acid and glycerol molecules. Expression of LPL increased about 11-fold. In early lactation, cows are typically in a negative energy balance and the increased energy requirement is compensated by mobilization of fatty acids from adipose tissues. Fatty acids obtained through the hydrolysis of circulating triacylglycerols by mammary LPL represent a significant source of milk fatty acids (Bell 1995). Consistently, expression of the very low density lipoprotein receptor (VLDL-R2), which binds and internalizes triglyceride-rich lipoproteins, also increased more than three-fold. Another gene, ecto-5'-nucleotidase, is a phosphatidylinositol anchored membrane structure considered to be the key enzyme in the formation and release of the adenosine nucleotides from ATP. Adenosine, a neuromodulator, is known to exert a multitude of physiological effects including vasodilation and stimulation of angiogenesis (Spychala 2000). These effects may potentially play an important role in nutrient supply to the mammary gland. However, expression of nitric oxide synthase trafficker (NOSTRIN), a protein facilitating internalization of nitric-oxide synthase (eNOS) (Icking et al. 2005; Schilling et al. 2006) and thus reducing another vascular mediator nitric oxide (NO), was increased, raising the possibility that NO may not play a role in stimulation of blood flow in the mammary gland in early lactation as previously suggested by Lacasse et al. (1996) and Prosser et al. (1996).

Nutrient uptake may play a rate-limiting role in milk synthesis. During the onset of lactation, mammary requirements of amino acids, glucose, and other nutrients for milk synthesis to surge dramatically. It has been estimated that mammary uptakes of glucose, amino acids, and fatty acids in Holstein cows are more than 1.8, 1.4, and 1.2 kg per day at 4 days postpartum, respectively (Bell 1995). Thus, it is not surprising to see that more than 10% of up-regulated transcripts are associated with the transport processes of amino acids, glucose, fatty acids, and ions. Glucose is the major precursor for synthesis of lactose, which controls milk volume by maintaining the osmolarity of milk. Mammary uptake of glucose increases ninefold on the day after parturition from day 7 to day 9 prepartum and fivefold from day 2 prepartum in the goat (Davis et al. 1979). GLUT1 is the major glucose transporter in the bovine mammary gland (Zhao et al. 1996; Zhao et al. 1993; Zhao et al. 2004). Our data here showed about twofold increases of both GLUT1 mRNA and protein at the onset of lactation and GLUT1 protein is mainly located in the plasma membrane of the mammary epithelial cells in lactation. In

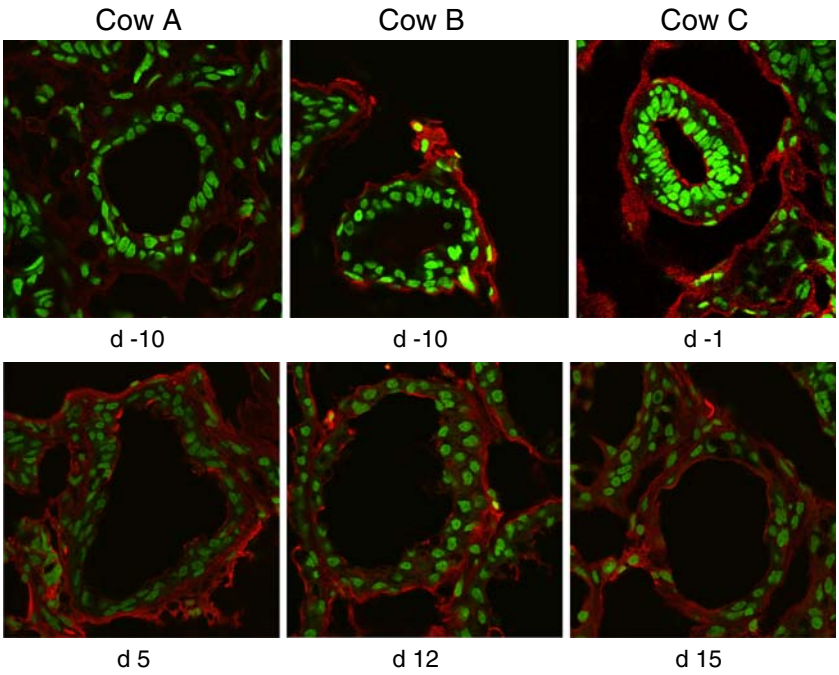

Fig. 4 Immunofluorescent staining of GLUT1 in the mammary gland of 3 Holstein cows around parturition using anti-GLUT1 polyclonal antibody and AlexaFluor 568-conjugated secondary antibody (in red). The biopsy date of each tissue sample is indicated by the day relative to parturition. The *top* and *bottom* rows are tissue samples of the mammary gland in late pregnancy and early lactation, respectively. The samples in each column were taken from the same animal. All tissues were counterstained with Sytox nuclear stain (in *green*). Magnification=630

addition, our previous study showed a hundred-fold increase in GLUT1 mRNA from 40 days before parturition to 7 days after parturition and a gradual increase in GLUT1 gene expression during this period (Zhao et al. 2004). Our microarray data indicated that the expression of amino acid and ion transporters may have a larger increase at the onset of lactation.

The increased expression of genes associated with metabolism and transport activities is coupled with overwhelmingly decreased expression of genes involved in cell division, cell cycle and their related processes, such as microtubule-based process, DNA replication, chromosome organization, and biogenesis. During pregnancy, the mammary gland undergoes major structural development and functional differentiation to produce milk. This is accomplished by early-stage ductal morphogenesis and late proliferative phase of alveolar morphogenesis (Hovey et al. 2002; Neville et al. 2002; Tucker 2000). In many species, the mammary growth continues into early lactation, peaking at day 5 in dairy goats (Anderson et al. 1981). Our microarray data indicated that in cows the mammary growth at days 5 to 14 in lactation should be much slower than in late pregnancy or be halted due to the decreased expression of genes involved in cell division. This is consistent with the observation that the cow mammary cell proliferation rate, as determined by the fraction of alveolar nuclei staining for Ki-67, is highest in late pregnancy (11% at day −14) while the rate is only 0.75% at day 14 of lactation, but the concentration of DNA is the highest at this stage (Sorensen et al. 2006). In addition, consistent to reduced epithelial cellular expansion, our array also showed that expression of extracellular matrix proteins of laminin and collagen decreased in early lactation.

Another group of genes, which were down-regulated at early lactation, was proteasomal genes and other related genes involved in proteolysis. This group of genes were also shown to have a decreased expression throughout pregnancy and lactation and an increased expression during involution in mouse mammary gland (Rudolph et al. 2003). It was speculated that the decline in proteasomal proteins is a functional adaptation of the mammary gland to conserve biosynthetic processes activated during lactation and/or to require less mRNA to replenish degraded protein (Rudolph et al. 2003).

Several calcium-binding proteins were also shown to be down-regulated in early lactation. This may spare calcium for its binding to milk proteins, such as caseins. In addition, as calcium is an important second messenger in cell signaling processes, this down-regulation may indicate that the signaling pathways through calcium play a role in regulating mammary cell growth and functioning in early lactation. In line with this hypothesis, the delta-like homolog (*Drosophila*) has been found to be involved in the differentiation of several cell types (Ruiz-Hidalgo et al. 2002).

Mammary gland development and its underlying gene expression is tightly regulated by reproductive hormones (Li et al. 2006). Similarly, initiation of lactogenesis is controlled by several hormones, which can either be inhibitory or stimulatory in their effects. Progesterone has a stimulatory effect on alveolar proliferation and lactogenesis stage I but a pronounced inhibitory effect on lactogenesis stage II. Its levels rapidly decrease from late

pregnancy to early lactation while the lactogenic hormone prolactin sharply increases in the same period (Neville et al. 2002). These hormones may play a major role in regulating gene expression changes detected in our microarray analysis. For example, prolactin has been shown to stimulate LPL and GLUT1 expression in the mammary gland (Hang and Rillema 1997; Hudson et al. 1997). The parathyroid hormone-like hormone (PTHLH) showed dramatically increased expression in early lactation. This hormone may also have a critical role as a local regulator. However, our microarray did not detect significant expression changes in genes directly linked to prolactin or progesterone signaling, such as the prolactin and progesterone receptors and STAT-5 (Yang et al. 2000a, b). Expression changes of these genes may occur at earlier stages or may not be required because the signaling factors can also be activated through phosphorylation and other means (Liu et al. 1996). Several other cell signaling factors, however, did show significant up-regulation in early lactation, implying that they may have regulatory roles in lactation. For example, Rab18 is a Ras-related protein and a member of a large family of small GTPase proteins that are involved in recognition processes between intracellular vesicles and their target membranes, and therefore are crucial regulators of endocytosis, exocytosis, and vesicle trafficking (Zerial and McBride 2001). These are important processes in milk secretion. In addition, several G-protein-associated proteins are down-regulated during lactation, suggesting a down-playing of G-protein signaling in early lactation.

In summary, in the mammary gland, the onset of lactation is supported by increased gene expression in metabolism and transporter activities and is coupled with decreased gene expression in cell proliferation and proteolytic activities.

Acknowledgements We gratefully acknowledge the assistance of Pamela Bentley, Emma Wall, and Jeffrey White, Department of Animal Science, University of Vermont, in the mammary tissue biopsies. We also acknowledge the excellent technical support of Scott Tighe and Timothy Hunter, Vermont Cancer Center, in microarray analysis. This project was supported by National Research Initiative Competitive Grant no. 2007-35206-18037 from the USDA Cooperative State Research, Education, and Extension Service (to FQZ), the University of Vermont Agriculture Experiment Station (to FQZ) and Alberta Agriculture of Canada (to JJK).

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