Journal of Dairy Research

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Functional analysis of the dairy cow mammary transcriptome between early lactation and mid-dry period

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Research Article

Cite this article: Lin Y, Lv H, Jiang M, Zhou J, Song S and Hou X (2019). Functional analysis of the dairy cow mammary transcriptome between early lactation and mid-dry period. *Journal of Dairy Research* **86**, 63–67. https://doi.org/10.1017/S0022029919000049

Received: 4 April 2018
Revised: 17 November 2018
Accepted: 30 December 2018
First published online: 7 February 2019

Keywords:
Differential gene expression; dry period; lactation; mammary gland

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Abstract

In this research communication we used digital gene expression (DGE) analysis to identify differences in gene expression in the mammary glands of dairy cows between early lactation and the mid-dry period. A total of 741 genes were identified as being differentially expressed by DGE analysis. Compared with their expression in dry cows, 214 genes were up-regulated and 527 genes were down-regulated in lactating cow mammary glands. Gene Ontology analysis showed that lactation was supported by increased gene expression related to metabolic processes and nutrient transport and was associated with decreased gene expression related to cell proliferation. Pathway mapping using the Kyoto Encyclopedia of Genes and Genomes showed that 579 differentially expressed genes had pathway annotations related to 204 pathways. Metabolic pathway-related genes were the most significantly enriched. Genes and pathways identified by the present study provide insights into molecular events that occur in the mammary gland between early lactation and mid-dry period, which can be used to facilitate further investigation of the mechanisms underlying lactation and mammary tissue remodeling in dairy cows.

During lactation, the mammary gland of dairy cows undergoes dramatic physiological and metabolic changes in support of lactogenesis and then milk secretion. When milk removal ceases, the dairy cow requires a 6 to 8-week dry period. The dry period is a non-lactating state initiated between lactation cycles that allows for optimal milk yield in the subsequent lactation period through the turnover of older, senescent mammary epithelial cells and their replacement with new, active cells (Capuco et al. 1997). The transition from lactation to the dry period is mediated by large-scale changes in gene expression. Limited studies have used quantitative real-time PCR (qPCR) and transcriptome analysis to evaluate the molecular events occurring in the mammary gland between lactation and early involution (Dado-Senn et al. 2018), but none have rigorously compared mammary glands during the peak stage of lactation and the mid-dry period to evaluate how each physiological stage is supported by broad-spectrum shifts in gene expression.

Digital gene expression (DGE), a tag-based transcriptome sequencing method, is a powerful approach to evaluate global gene expression in tissues or cells of interest in response to changes in physiological state ('t Hoen et al. 2008). DGE has been applied to evaluate the molecular events occurring during the onset of lactation in the mammary gland of dairy cows (Gao et al. 2013). Here we used DGE to examine global gene expression in dairy cow mammary glands between the peak stage of lactation and the mid-dry period. Genes and pathways identified by the present study will provide fundamental insights into tissue remodeling during the dry period, as well as a better understanding of milk production during lactation.

Materials and methods

All experimental procedures involving animals were approved by Northeast Agricultural University (China). Six multiparous Holstein cows with similar genetic backgrounds were used in the study. Among them, three were lactating cows during the peak lactation stage (after their third parity, milk yield = 33·9 ± 2·1 kg/d), and three were pregnant and dried off after being milked for 310 d (milk yield = 33·2 ± 1·4 kg/d for the previous lactation period). The lactating cows were slaughtered at 90 DIM, and the dry cows were slaughtered at 30 d after dry off. Approximately 50 g of mammary parenchymal tissue was collected from the mid-alveolar region of each cow.

Extraction of total RNA from 100 mg of homogenized mammary tissue from each cow was performed using Trizol reagent (Invitrogen, CA) and was purified using RNeasy mini kit

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Table 1. Top 50 of up-regulated or down-regulated transcripts in the mammary glands of dairy cows during early lactation *vs.* mid-dry period

Gene symbol	GenBank accession	Gene description	SFC	*P* value
Up-regulated				
FAM159B	NM_001100373·2	Family with sequence similarity 159, member B	34·60	$1·00 \times 10^{-12}$
GPC5	NM_001102070·1	Glypican 5	33·63	$4·11 \times 10^{-09}$
TECTB	NM_001083733·1	Tectorin beta	32·87	$8·01 \times 10^{-07}$
IARS2	NM_001206795·1	Isoleucyl-tRNA synthetase 2, mitochondrial	31·80	0·000504
GUK1	NM_001159412·1	Guanylate kinase 1	31·63	0·000748
HS3ST5	NM_001076215·1	Heparan sulfate-glucosamine 3-sulfotransferase 5	31·47	0·001708
STMN4	NM_001075580·2	Stathmin 4	30·88	0·017199
CALB1	NM_001076195·1	Calbindin 1	8·79	$1·18 \times 10^{-25}$
LALBA	NM_174378·2	Lactalbumin alpha	8·21	$6·52 \times 10^{-28}$
GLYCAM1	NM_174828·2	Glycosylation-dependent cell adhesion molecule 1	7·52	$4·65 \times 10^{-25}$
GP2	NM_001075950·2	Glycoprotein 2	7·41	$7·32 \times 10^{-24}$
SLC34A2	NM_174661·2	Solute carrier family 34 member 2	7·38	$1·38 \times 10^{-16}$
TUBA1C	NM_001034204·1	Tubulin, alpha 1c	7·28	$1·23 \times 10^{-16}$
CSN1S1	NM_181029·2	Casein alpha s1	6·55	$5·39 \times 10^{-21}$
CSN2	XM_010806178·1	*Bos taurus* casein beta	6·52	$6·57 \times 10^{-21}$
PTHLH	NM_174753·1	Parathyroid hormone like hormone	6·48	$2·58 \times 10^{-19}$
BDA20	NM_174761·2	Major allergen BDA20	6·24	$1·19 \times 10^{-09}$
ALOX15	NM_174501·2	Arachidonate 15-lipoxygenase	6·20	$2·20 \times 10^{-14}$
BOLA-DQA1	NM_001013601·3	Major histocompatibility complex, class II, DQ alpha, type 1	6·19	$4·45 \times 10^{-14}$
TTC36	NM_001040515·2	Tetratricopeptide repeat domain 36	6·17	$1·54 \times 10^{-09}$
PAEP	NM_173929·3	Progestagen-associated endometrial protein	5·79	$6·92 \times 10^{-18}$
SPINK4	NM_001114522·1	Serine peptidase inhibitor, Kazal type 4	5·77	$9·33 \times 10^{-11}$
BTN1A1	NM_174508·2	Butyrophilin, subfamily 1, member A1	5·77	$1·69 \times 10^{-17}$
TMOD4	NM_001075215·1	Tropomodulin 4	5·44	$1·25 \times 10^{-12}$
SCD	NM_173959·4	Stearoyl-CoA desaturase	5·34	$5·47 \times 10^{-16}$
MYBPC1	NM_001110773·1	Myosin binding protein C, slow type	5·31	$9·98 \times 10^{-07}$
ASB11	NM_001034413·2	Ankyrin repeat and SOCS box containing 11	5·22	$2·89 \times 10^{-15}$
STATH	NM_174752·2	Statherin	5·14	$1·41 \times 10^{-09}$
LPL	NM_001075120·1	Lipoprotein lipase	5·10	$7·34 \times 10^{-15}$
SLC38A3	NM_001040486·1	Solute carrier family 38 member 3	4·94	$1·98 \times 10^{-10}$
Down-regulated				
LOC100125916	NM_001105487·1	Uncharacterized protein 100125016	−35·21	$2·83 \times 10^{-13}$
PNMT	NM_177505·3	Phenylethanolamine N-methyltransferase	−35·18	$3·20 \times 10^{-13}$
BPIFA1	NM_174426·3	BPI fold containing family A member 1	−34·13	$1·78 \times 10^{-09}$
TAP	NM_174776·1	Tracheal antimicrobial peptide	−33·63	$1·25 \times 10^{-08}$
LOC789175	NM_001114862·1	Beta-defensin 103B-like	−33·33	$1·03 \times 10^{-07}$
ORM1	NM_001040502·2	Orosomucoid 1	−33·22	$2·11 \times 10^{-07}$
VSTM1	NM_001101274·2	V-set and transmembrane domain containing 1	−33·15	$3·71 \times 10^{-07}$
LRRC25	NM_174688·4	Leucine rich repeat containing 25	−33·13	$4·50 \times 10^{-07}$
CCL3	NM_174511·2	Chemokine (C-C motif) ligand 3	−32·94	$1·51 \times 10^{-06}$
BTK	NM_001034589·1	Bruton tyrosine kinase	−32·92	$1·87 \times 10^{-06}$
CXCR1	NM_174360·2	Chemokine (C-X-C motif) receptor 1	−32·85	$2·32 \times 10^{-06}$

(*Continued*)

https://doi.org/10.1017/S0022029919000049 Published online by Cambridge University Press

Table 1. (*Continued.*)

Gene symbol	GenBank accession	Gene description	SFC	*P* value
PRR3	NM_001034554·1	Proline rich 3	−32·85	$2{\cdot}89 \times 10^{-06}$
BFSP2	NM_174248·3	Beaded filament structural protein 2	−32·83	$2{\cdot}89 \times 10^{-06}$
IL1R2	NM_001046210·2	Interleukin 1 receptor type 2	−32·77	$3{\cdot}62 \times 10^{-06}$
RSPO1	NM_001105621·1	R-spondin 1	−32·54	$1{\cdot}49 \times 10^{-05}$
CLEC11A	NM_001098054·1	C-type lectin domain containing 11A	−32·39	$4{\cdot}16 \times 10^{-05}$
SNAI1	NM_001112708·1	Snail family transcriptional repressor 1	−32·38	$4{\cdot}16 \times 10^{-05}$
PTP4A3	NM_001083766·1	Protein tyrosine phosphatase type IVA	−32·28	$7{\cdot}15 \times 10^{-05}$
VASH1	NM_001206803·1	Vasohibin 1	−32·28	$7{\cdot}15 \times 10^{-05}$
CDCA3	NM_001035449·2	Cell division cycle associated 3	−32·28	$7{\cdot}15 \times 10^{-05}$
VPREB2	XM_002694636·1	Pre-B lymphocyte gene 2	−32·19	$9{\cdot}46 \times 10^{-05}$
CGREF1	NM_001045977·2	Cell growth regulator with EF-hand domain 1	−32·17	0·000126
C1QTNF6	NM_001101872·2	C1q and TNF related 6	−32·16	0·000126
MMP7	NM_001075130·1	Matrix metallopeptidase 7	−32·14	0·000168
CDYL2	NM_001163785·1	Chromodomain Y like 2	−32·12	0·000168
ACOX1	NM_001035289·3	Acyl-CoA oxidase 1	−32·09	0·000168
CCDC51	NM_001015619·1	Coiled-coil domain containing 51	−32·07	0·000226
FAM104A	NM_001035460·2	Family with sequence similarity 104 member A	−32·03	0·000226
NFE2	NM_001014923·1	Nuclear factor, erythroid 2	−31·92	0·000416
CCNB1	NM_001045872·1	Cyclin B1	−31·91	0·000416

SFC, signed fold change.
The gene list is part of differentially expressed genes in DGE analysis.

(Qiagen, Netherlands). Then a total of 6 μg extracted RNA per cow was used for DGE library construction (details in Supplementary File 1). Each library was sequenced on an Illumina HiSeq 2000 system (BGI, Beijing). Clean tags were generated by removing empty tags, low-quality tags, and tags with only one copy number. All clean tags were aligned to the bovine reference genome (UMD3·1, ftp://ftp.ensembl.org/pub/release-79/fasta/bos_taurus/dna/), and unambiguous tags were annotated. For gene expression analysis, the number of unambiguous clean tags for each gene was calculated and then normalized to the number of transcripts per million clean tags.

A statistical analysis of the frequency of each tag in the different DGE libraries was performed to compare the differences in gene expression. A false discovery rate of ≤0·001 and an absolute value of log 2 ratio ≥ 1 were used as cutoffs to judge the significance of gene expression differences. qPCR analysis was used to validate the DGE results.

The selected genes with significant differential expression in lactating cows and dry cows were analyzed in the context of Gene Ontology (GO) terms biological process, molecular function, and cell component (http://www.geneontology.org/). A corrected *P* value of <0·05 was selected as the threshold for significant enrichment of the gene sets. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway visualization for the comparison of lactating cows and dry cows was performed using the application KeggArray available in the KEGG website at http://genome.jp/kegg/. Pathways with $P < 0{\cdot}05$ were considered strongly enriched in DGE.

Results and discussion

We identified 741 transcripts that were significantly differentially expressed between the early lactation period and the mid-dry period. Of these, 214 genes were up-regulated and 527 genes were down-regulated in the mammary glands of lactating cows compared with those of dry cows (Table 1). Validation of a subset of these genes by qPCR (online Supplementary Fig. S1, Table S1) indicated that our DGE profiling analysis is of high quality and is biologically relevant.

To identify the biological pathways that were highly enriched in mammary glands of lactating cows *vs.* dry cows, all the DEGs were mapped to the terms in the KEGG database and compared with the whole transcriptome background. Pathway mapping using the KEGG showed that 579 DEGs had pathway annotations that were related to 204 pathways. The specific enrichment of genes was observed in the metabolic pathway in the mammary glands of lactating cows compared with those of the dry cows (Fig. 1a), which is consistent with the increased metabolic activity of the mammary gland during lactation.

The DEGs were then analyzed in the context of the GO categories biological process, molecular function, and cellular component. This analysis revealed that the majority of these DEGs are associated with the cellular process term in the biological process ontology category, with the cell and cell part terms in the cellular component ontology category, and with the binding term in the molecular function ontology category (Fig. 1b).

Consistent with the KEGG results, the GO analysis revealed that one of the major GO biological processes is metabolic

https://doi.org/10.1017/S0022029919000049 Published online by Cambridge University Press

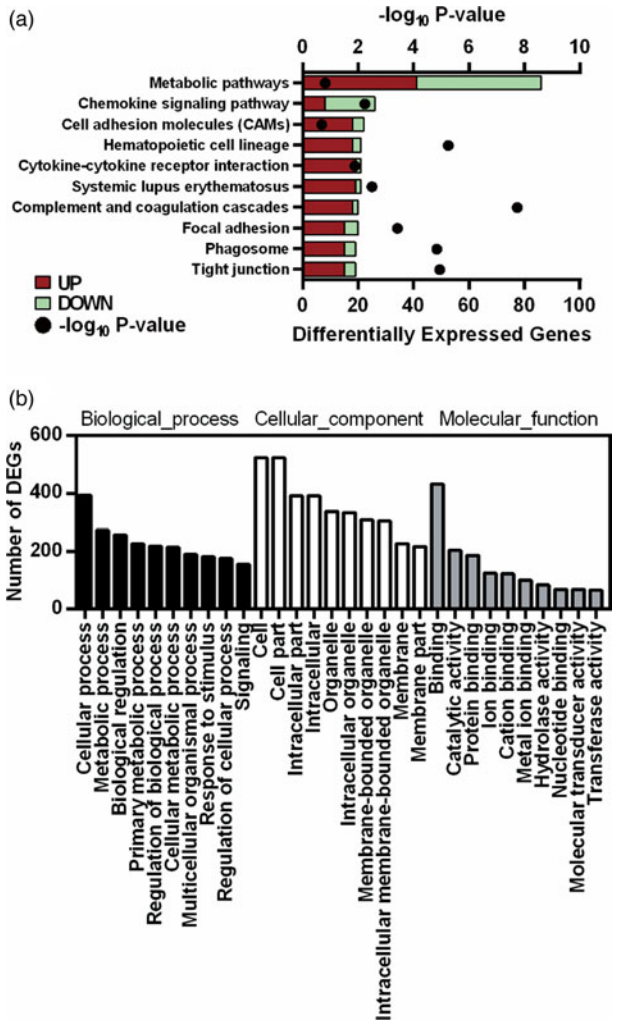

Fig. 1. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) analyses of differentially expressed genes (DEG) in mammary glands of dairy cows between the early lactation period and mid-dry period. (a) The significantly enriched KEGG pathways in dairy cow mammary gland during early lactation *vs.* mid-dry period. *x*-axis, the total significance of enrichment ($-\log_{10}$ *P*-value) and the number of DEG within each pathway. Color version available online. (b) Significantly enriched GO terms in the biological process, cellular component and molecular function (top ten GO terms) categories, respectively. *y*-axis, the number of DEG.

process. Among the 214 up-regulated genes in lactating mammary tissues as compared with dry cow mammary tissues, 58 were associated with metabolic process (online Supplementary Table S2). During lactation, the mammary epithelial cells synthesize and secrete copious amounts of carbohydrate, protein, and lipid. Our DGE data showed that the expression of milk protein genes increased remarkably in mammary glands of lactating cows as compared with dry cows (online Supplementary Table S3). We also observed that genes associated with fatty acid (FA) uptake from blood (*LPL, CD36*), intracellular FA trafficking (*FABP3*), long-chain (*ACSL1*) and short-chain (*ACSS2*) intracellular FA activation, *de novo* FA synthesis (*ACACA*, *FASN*), FA desaturation (*SCD*), triacylglycerol synthesis (*GPAT4*, *GPAM*), and transcription regulation (*PPARGC1A*) were up-regulated in mammary glands of lactating cows as compared with dry cows. This is consistent with the findings of Bionaz & Loor (2008), which indicated that lactation induced increased mRNAs of genes involved in the *de novo* synthesis of FAs, FA desaturation, and the synthesis of triacylglycerol.

During lactation, mammary epithelial cells secrete large quantities of milk. Casein micelles are secreted by exocytosis of Golgi-derived secretory vesicles, whereas lipids are released as milk fat globules by budding of the apical plasma membrane of mammary epithelial cells (Truchet et al. 2014). In this study, GO analysis revealed that several highly expressed genes were associated with vesicle-mediated transport in lactating mammary tissues, which reflects the increased secretion activity of the mammary gland. Highly expressed vesicle-related genes included ras-related protein Rab-18 (*RAB18*), double C2-like domain-containing protein gamma (*DOC2G*), and vesicle-associated membrane protein 1 (*VAMP1*). In addition, we also detected higher expression of the cell death activator *CIDEA* in lactating cows as compared with dry cows, which functions in milk lipid secretion in mice (Wang et al. 2012). These genes may be involved in maintaining the secretory activity of mammary epithelial cells during lactation and may, therefore, serve as target genes for manipulation of lactation performance.

We also observed that 32 of the up-regulated genes (of 214 transcripts) in mammary tissues during early lactation as compared with mid-dry period were associated with GO category involved in transport (online Supplementary Table S4). During lactation, mammary gland requirements for FAs, amino acids, glucose, and other nutrients for milk synthesis surge dramatically. The up-regulated genes in lactation that are involved in the FA transport process included *FABP3* and *CD36*; a gene involved in glucose transport was tribbles pseudokinase 3 (*TRIB3*), and those involved in the amino acid transport process were solute carrier family 38 member 3 (*SLC38A3*), solute carrier family 7 member 5 (*SLC7A5*) and solute carrier family 22 member 16 (*SLC22A16*). *SLC7A5* is a sodium-independent transporter mediating the cellular uptake of essential amino acids. Our data showed an increase in *SLC7A5* mRNA that was approximately twofold in lactating cows relative to dry cows. This is in accord with previous data showing that in dairy cows, a large up-regulation in expression of *SLC7A5* was observed during lactation relative to pregnancy (Bionaz & Loor, 2011). Therefore, targeted expression of *SLC7A5* may be a useful intervention to improve milk protein synthesis in dairy cows.

With respect to cell proliferation, the GO analysis showed ≥30 significantly down-regulated genes in lactating cows relative to dry cows (online Supplementary Table S5). The mammary gland is a dynamic organ that undergoes cycles of cell proliferation, differentiation, and apoptosis during adult life. In pregnant dry cows, there is a significant increase in cell proliferation from the early to late dry period, as quantified by in vitro incorporation of [^{3}H]-thymidine (Capuco et al. 1997). In our study, the higher expression of genes involved in proliferation in dry cows indicates that, during the dry period, the epithelial compartment is replacing old senescent cells with new cells and cells are preparing to synthesize proteins and lipids that are necessary for lactation. The mammary epithelium is closely linked to the stroma through the basement membrane extracellular matrix. Stromal cells influence epithelial cell behavior by secreting growth factors. Insulin-like growth factor 1 (IGF1) produced by stromal cells acts in a paracrine fashion on mammary epithelial cells to stimulate cell proliferation (Collier et al. 2012). Fibroblast growth factor 1 (FGF1) likely functions as a paracrine mitogen for mammary

https://doi.org/10.1017/S0022029919000049 Published online by Cambridge University Press

epithelial cells, and its highest level of expression is in primiparous heifers during involution (Sinowatz et al. 2006). In our study, we observed an increase in the expression of *IGF1*, *FGF1*, and other growth factors during the dry period. This is consistent with a role for these growth factors in the stimulation of cell proliferation.

In conclusion, we demonstrated that 741 transcripts were significantly differentially expressed in mammary glands of dairy cows between early lactation period and mid-dry period. In dairy cows, lactation is supported by increased gene expression related to metabolic processes and nutrient transport. Mammary tissue remodeling during the mid-dry period is supported by increased gene expression related to cell proliferation. The identification of enriched genes and pathways in the present study will provide fundamental insights into tissue remodeling during the dry period, as well as a better understanding of lactation.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029919000049.

Financial support. This work was supported by grants from the University Nursing Program for Young Scholars with Creative Talents in Heilongjiang Province (UNPYSCT-2015010, China).

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