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ORIGINAL ARTICLE

Transcriptomic profiles of the bovine mammary gland during lactation and the dry period

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Abstract

The initiation and maintenance of lactation are complex phenomena governed by biochemical and endocrine processes in the mammary gland (MG). Although DNA-based approaches have been used to study the onset of lactation, more comprehensive RNA-based techniques may be critical in furthering our understanding of gene alterations that occur to support lactation in the bovine MG. To further determine how gene profiles vary during lactation compared with the dry period, RNA-seq transcriptomic analysis was used to identify differentially expressed genes (DEG) in bovine MG tissues from animals that were lactating and not lactating. A total of 881 DEG (605 upregulated and 276 downregulated) were identified in MG of 3 lactating Chinese Holstein dairy cows versus the 3 dry cows. The subcellular analysis showed that the upregulated genes were most abundantly located in "integral to membrane" and "mitochondrion," and the top number of downregulated genes existed in "nucleus" and "cytoplasm." The functional analysis indicated that the DEG were primarily associated with the support of lactation processes. The genes in higher abundance were most related to "metabolic process," "oxidation-reduction process," "transport" and "signal transduction," protein synthesis-related processes (transcription, translation, protein modifications), and some MG growth-associated processes (cell proliferation/cycle/apoptosis). The downregulated genes were mainly involved in immune-related processes (inflammatory/immune/defense responses). The KEGG analysis suggested that protein synthesis-related pathways (such as protein digestion and absorption; protein processing in endoplasmic reticulum; and glycine, serine, and threonine metabolism) were highly and significantly enriched in the bovine MG of lactating cows compared to dry cows. The results suggested that the dry cows had decreased capacity for protein synthesis, energy generation, and cell growth but enhanced immune response. Collectively, this reduced capacity in dry cows supports the physiological demands of the next lactation and the coordinated metabolic changes that occur to support these demands. A total of 51 identified DEG were validated by RT-PCR, and consistent results were found between RT-PCR and the transcriptomic analysis. This work provides a profile of gene-associated changes that occur during lactation and can be used to facilitate further investigation of the mechanisms underlying lactation in dairy cows.

Keywords Lactation · Mammary gland · Transcriptomic · Dairy cows

Introduction

Lactation initiation and maintenance are a series of complex biological processes in the mammary gland (MG), including dramatic physiological and metabolic changes that occur during the transition from pregnancy to lactation (Bellmann 1976, Hurley 1989). Because of the degree of changes that have to occur, a dry period of appropriate length primes the cow for the subsequent lactation, and management through the transition from the dry period to lactation can directly affect MG development and milk

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production (Drackley 1999). To better understand factors affecting this transition, studies have extensively explored the functional development of the MG at genetic, physiological, and morphologic levels (Hurley 1989, McManaman and Neville 2003).

The metabolic and biological changes in bovine MG related to lactation have been explored through several different approaches. For example, quantitative PCR was used to identify a limited number of gene networks involved in bovine mammary protein and lipid synthesis during lactation (Bionaz and Loor 2008, 2011). Additionally, DNA microarrays have detected MG gene alterations during the onset of lactogenesis and their functional relevance to support the transition from pregnancy to lactation (Finucane et al. 2008). Although DNA microarrays are useful, they may only identify expression changes of static genes (Kumar et al. 2016) underlying the lactation-related processes in dairy cows. As such, more advanced approaches have been adopted. For example, proteomics has been used to assess coordinated shifts in the MG associated with onset of lactation. In a recent 2D-proteomic study from Dai et al. (2017b), a total of 60 differentially expressed proteins (DEP) were detected in the mammary gland of the lactating dairy cows compared with the dry cows. Although several techniques have been applied, a very limited genes or proteins have been detected in studies comparing tissue collected during different lactation-related periods. RNA-sequencing is acknowledged as a sensitive, broad-spectrum detection tool for identifying dynamic gene profiles underlying molecular and cellular processes (Ozsolak and Milos 2011). The rapid development of RNA-sequencing has made it feasible to conduct large-scale whole transcriptome sequencing projects, thereby providing deeper knowledge of transcriptomic regulation (Jiang et al. 2015). Although RNA-sequencing has been successfully applied to study the whole transcriptome of bovine mammary glands under different conditions (Cui et al. 2014, Hosseini et al. 2013, Ibeagha-Awemu et al. 2016), studies have not thoroughly compared the dry and lactating mammary glands to evaluate how physiological stage is supported by broad-spectrum shifts in gene expression.

The objective of this study was to more comprehensively explore the gene changes that occur between the MG of lactating and dry dairy cows by using RNA-seq transcriptomic analysis of MG total RNA obtained from dairy cows during lactation and the dry period. Because a major shift between the lactating and dry periods is the production of milk, it is likely that this comparative analysis will also help to identify some key genes closely related to milk synthesis.

Materials and methods

Animals, experimental design, and sample collection

All the experimental protocols were approved by the Animal Care Committee, Zhejiang University, Hangzhou, P. R. China, and all animal handling procedures used in this study were in compliance with the Guidelines of China for Animal Care and conducted in accordance with the approved protocols. The six cows used were multiparous China Holstein dairy cows and were fed a diet including 23% alfalfa hay and 7% Chinese wild rye hay with a forage-to-concentrate ratio of 45:55 [dry matter (DM) basis]. The chemical composition of the individual forages and the experiment procedures were described in the study of Wang et al. (2014). Of the cows in the dry period group, one was 5 years old and two were 4 years old (all were non-lactating in the early stage of pregnancy). In the mid-lactation group, one cow was 4 years old and 92 days in milk (DIM), one was 5 years old and 98 DIM, and the other one was 6 years old and 118 DIM. The MG samples were collected immediately at slaughter. After removing the connective and adipose tissue, approximately 50 g of MG tissue collected from each cow was washed three times with ice-cold PBS, sterilized with 75% ethyl alcohol, snap-frozen in liquid N_2, and stored at − 80 °C until further analysis.

RNA extraction

The total RNA extraction from 100 mg homogenized MG tissue from each animal was performed using Trizol reagent (Invitrogen, Carlsbad, CA). The obtained RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE) and then purified by an RNA 6000 Nano LabChip Kit (Agilent, CA, USA). With an integrity number (RIN) more than 7.0 and a ratio of 28S/18S ranging from 1.5 to 2.6, 5 μg of the extracted RNA was used for RNA-seq library construction.

RNA-seq library construction and RNA sequencing

A Truseq® Stranded mRNA Sample Preparation kit (Illumina, San Diego, CA, USA) was used to prepare the RNA library using 1 μg extracted total RNA following the manufacturer's instructions. The constructed libraries were measured using an Agilent 2200 TapeStation and a Qubit 2.0 Fluorimeter to ensure that the average insert size for paired-end libraries was 400 bp (± 50 bp) and that the concentration was adequate for sequencing. The RNA sequencing was performed on an Illumina 2500 sequencing system (Illumina, San Diego, CA, USA) at LC Biotech (Hangzhou, China).

Differential gene expression analysis

The bovine reference genome sequences (UMD3.1) were downloaded from NCBI (ftp://ftp.ensembl.org/pub/release-79/fasta/bos_taurus/dna/). After removing low-quality reads and adapter sequences, clean reads were aligned to the bovine reference genome using Bowtie version 0.12.7 included in TopHat 2.0.9 (http://ccb.jhu.edu/sofware/tophat/index.shtml), ensuring reads were shorter than 20 bp. Only two mismatches were allowed. To construct the transcriptome, the mapped reads were assembled de novo using Cufflinks version 1.2.1 (http://cole-trapnell-lab.github.io/cufflinks/). All transcripts were required to be > 200 bp in length. The assembled transcripts were annotated using the Cuffcompare program from the Cufflinks package (Trapnell et al. 2014). According to the annotation of the bovine genome sequence (UMD3.1), the known transcripts were identified. Expression levels of all transcripts were estimated as FPKM (fragments per kilobase of exon per million fragments mapped) using the Cuffdiff program from the Cufflinks package. The sum of all the isoforms with qualified FPKM was used as the FPKM of each gene. Genes were removed from the analysis if the FPKM in all samples was 0. The total sum of the gene FPKM was calculated for each sample in the two groups. The FPKM values were then normalized to FPKM in 1 million of the summed FPKM. Finally, 1 was added to all normalized FPKM. Upregulation of DEG was defined at a threshold fold change (FC(Lactation/Non-lactation)) ≥2 and $P < 0.05$, and downregulation of DEG was defined at a threshold (FC(Lactation/Non-lactation)) ≤ 0.5 and $P < 0.05$.

Bioinformatic analysis

Functional annotations were performed using the Gene Ontology (GO) annotation software (ftp://ftp.ncbi.nih.gov/gene/DATA/gene2go.gz). The metabolic pathway analysis was processed using R software packages included in the R-project (R version 3.2.3) (Team RC 2014, Walter et al. 2015) according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/). The GO and KEGG pathway enrichment statistics were performed by Fisher's exact test with a cut-off P value of less than 0.05 considered as significant for both GO terms and pathways.

Quantitative reverse transcription PCR (qRT-PCR) analysis

Total RNA extracted from the MG tissue was reverse transcribed for cDNA synthesis using a PrimeScript^{RT} Reagent Kit with gDNA Eraser (Takara, Tokyo, Japan) following the manufacturer's instructions. The qRT-PCR was performed in triplicate using the Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The 20 μL reaction included 50 ng of reverse transcription product, 40 nM of each forward and reverse primers [Table S1, designed by Primer 5 software (Premier Biosoft International, Palo Alto, CA)], and the SYBR Premix Taq (Takara, Tokyo, Japan). The running program was 1 cycle of 95 °C for 30 s plus 40 cycles of amplification at 95 °C for 5 s and 58 °C for 30 s, followed by an additional 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C to generate the melt curve. The relative gene expression values were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The relative expression of target genes was normalized to the expression of β-actin and ribosome protein 9 (RPS9). Data were statistically analyzed by the SAS software (SAS Institute, Car, NC, USA) using one-way ANOVA with Duncan's multiple range tests. $P < 0.05$ was considered as a significant difference.

Results

Differential transcriptomic profiles in the lactating and dry MG

The RNA extracted from MG tissues of lactating and dry cows were subjected to RNA-seq transcriptomic analysis, generating approximately 32 and 39 million raw reads for each (lactating and dry) library, respectively (Table S2). After removal of the low-quality reads, 32,416,688 and 38,940,178 pairs of clean reads were obtained in the two groups (Table S2). Among the two RNA-seq libraries, 77.9 and 86.0% of reads were mapped to a gene in the reference database (UMD3.1) with a unique match ratio of 70.8 and 75.7%, respectively (Table S2). Based on a P value ≤ 0.05 and |log2 FC| ≥ 1, a total of 881 DEG (605 upregulated and 276 downregulated) (Table S3) were identified in the bovine MG of lactating cows versus dry cows. Nearly half of the differentially expressed transcripts were uncharacterized. Additionally, the Pearson correlation coefficient (R) between the lactating and dry groups was 0.675 (Fig. S1), which indicated the transcripts in lactating group were highly consistent with those in the dry group.

Gene ontology analysis

Of the 881 DEG, 332 identified genes were classified into three classes (cellular processes, biological processes, and molecular function) using the GO annotation (http://david.abcc.ncifcrf.gov/home.jsp). Figure 1 demonstrates the subcellular analysis of the up- and downregulated genes using the GO and UniProt databases. The upregulated genes (Fig. 1a) in lactation were significantly (P value < 0.05) and abundantly located in "membrane" (30%), "organelle (28%), and intracellular (19%)." Notably, 57, 27 upregulated DEG were most

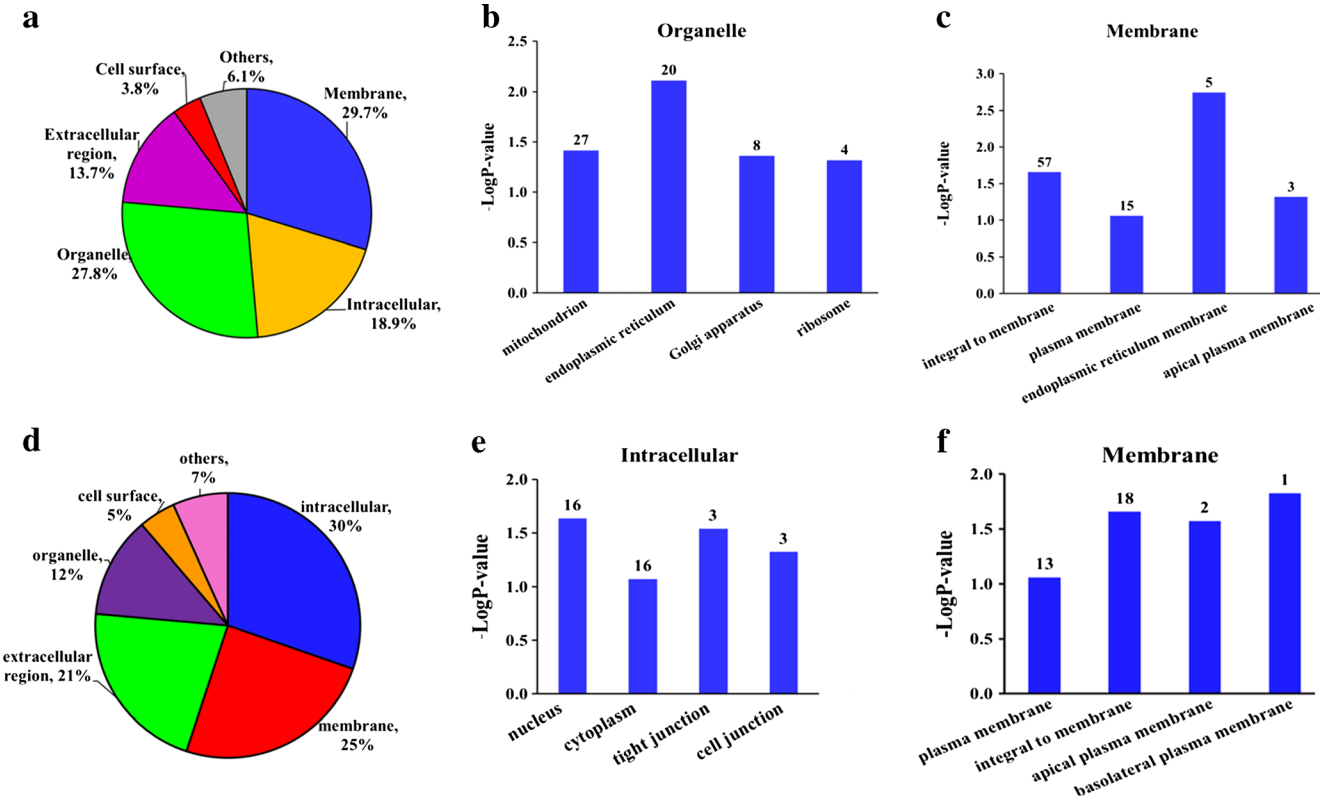

Fig. 1 Subcellular location of the upregulated (**a**) / downregulated (**d**) genes in the mammary gland of dairy cows during lactation versus non-lactation. The upregulated gene assigned to the category "membrane" (**b**) and "organelle" (**c**); the downregulated gene assigned to the category "intracellular" (**e**) and "membrane" (**f**). The number in the pie chart or within the bar graph indicated the number of differentially expressed genes assigned to each category

abundantly and significantly enriched in "integral to membrane" among the "membrane" categories (Fig. 1b), and in "mitochondrion" among the "organelle" class (Fig. 1c), respectively. In contrast, the downregulated genes (Fig. 1d) in lactation were significantly (P value < 0.05) and abundantly positioned in "intracellular (30%)," "membrane" (25%), and "extracellular region" (28%). Noticeably, 16 DEG in lower abundance (Fig. 1e) were primarily and significantly enriched in "nucleus" and "cytoplasm" among the "intracellular" class; and 18, 13 downregulated DEG (Fig. 1f) were also significantly enriched in "integral to membrane" and "plasm membrane" among the "membrane" categories, respectively.

Within the functional analysis (Fig. 2a and Table S4), most genes in higher abundance were enriched in the categories: "metabolic process" (21 DEG), "oxidation-reduction process" (19 DEG), "transport" (18 DEG), and "signal transduction" (15 DEG). Additionally, we also found that some upregulated genes were enriched in the categories: "positive regulation of transcription," "positive regulation of translation," and several protein modification processes including "protein processing" and "proteolysis." Notably, a small number of upregulated genes were enriched in several cell-growth-related processes involved in the maintenance of MG growth and health, which included "positive regulation of cell differentiation," "negative regulation of apoptotic process," and "positive regulation of cell cycle." In contrast, among biological processes enriched by the abundant downregulated members (Fig. 2b and Table S5) were "inflammatory response" (12 DEG), "immune response" (11 DEG), "defense response" (5 DEG), and "lipopolysaccharide (LPS)-mediated signaling pathway" (2 DEG), which were related to the immune processes of the dairy cows' body. Also, some genes in lower abundance were associated with the categories: "negative regulation of cell death," "apoptotic process," "negative regulation cell death," and "negative regulation of cell cycle." Moreover, several biological processes—"negative regulation of TOR signaling cascade" and "misfolded or incompletely synthesized protein catabolic process" were also assigned by several downregulated genes.

Key metabolic pathways analysis

The Fig. 3 and Table 1 showed the KEGG pathway analysis of DEG and several important protein synthesis-related pathways were identified as significantly enriched by some DEG. For example, 10, 4, 4, 4, 2 genes in higher abundance were enriched in the pathway of "protein digestion and absorption ($P = 0.0013$)" and "protein processing in

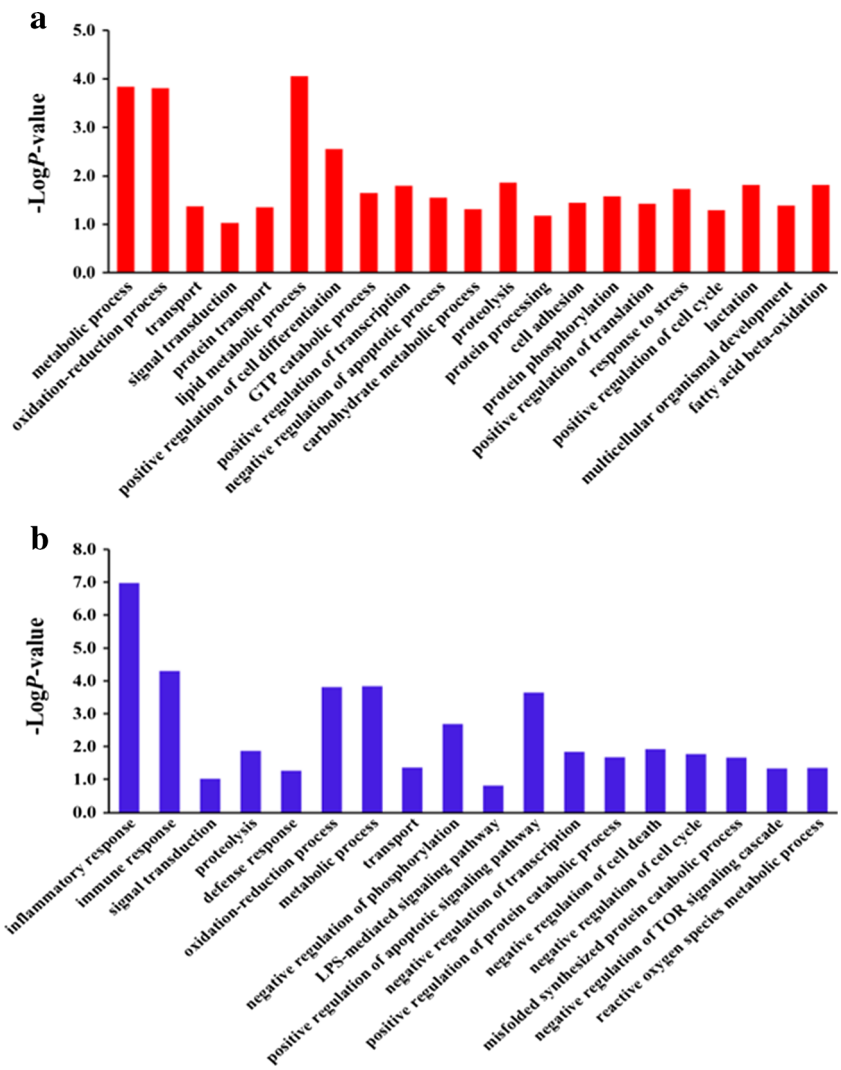

Fig. 2 Functional analysis of the upregulated (**a**) and downregulated (**b**) genes in the mammary gland of dairy cows during lactation versus non-lactation by gene ontology analysis. The *y*-axis panel shows the value of –Log (*P* value), the *x*-axis shows the functional categories of up−/downregulated genes

endoplasmic reticulum" ($P = 0.0287$), "ribosome" ($P = 0.0096$), "glycine, serine, and threonine metabolism" ($P = 0.0134$) and "aminoacyl-tRNA biosynthesis" ($P = 0.0097$), respectively. In addition, three downregulated genes were significantly enriched in the "mTOR signaling pathway" ($P = 0.0328$). Notably, with a *P* value less than 0.01, 9 and 13 downregulated DEG were enriched in "chemokine signaling pathway" and "cytokine-cytokine receptor interaction," respectively. Furthermore, several cell-growth-associated signaling pathways (such as "TGF-beta signaling pathway," "insulin signaling pathway," and "MAPK signaling pathway") were also significantly enriched by a small number of upregulated DEG.

Verification of DEG by qRT-PCR analysis

A total of 51 genes involved in organic nutrient metabolism (Fig. 4a), related to mammary health, or present in milk protein (Fig. 4b) were selected for qRT-PCR analysis. Among these genes, expression levels of 40 genes were significantly altered in the MG between the dry and lactating groups, and the abundance of 38 genes were consistent with expression patterns measured by RNA-seq. Furthermore, the correlation of mRNA expression level of the selected 51 genes by RNA-Seq and RT-PCR was relatively high ($r = 0.859$) (Fig. 5). The comprehensive view of the molecular mechanisms underlying milk production was summarized based on the transcriptomic data (Fig. 6 and Table S6). The regulatory parts proposed include two major sections (nutrient metabolism and mammary health) and six subsections—lactose synthesis, energy metabolism, lipid metabolism, amino acid/protein metabolism, mammary cell number, and immune response. Compared to our previous 2D-proteomic analysis of MG during lactation versus the dry period (Dai et al. 2017b), the functional level analysis of DEG suggests that transcriptomic analyses of the bovine MG might be more sufficient to characterize tissue functional responses and be reflective of shifts in specific genes.

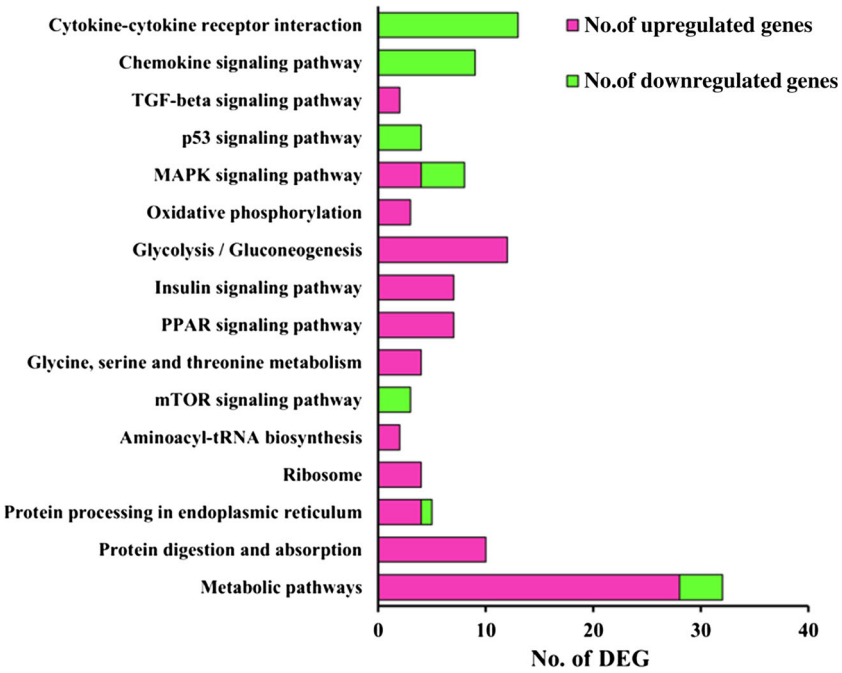

Fig. 3 KEGG enriched pathways of differentially expressed genes in the mammary glands dairy cows during lactation versus non-lactation. The bottom panel shows the number of DEG mapped to the pathway. The pink bars represent the enriched pathway of the upregulated genes, the green bars represent the enriched pathway of the downregulated genes

Discussion

To reduce the effect of diet on the mammary metabolisms of dairy cows, we used a relatively high-quality diet containing 23% alfalfa and 7% Chinese wild hay as forage. This diet was identical to the diet reported in the study from Dai et al. (2017a), which demonstrated that the MG from cows fed high-quality forage had more potential to enhance milk protein production and lower protein degradation compared to that from cows fed low-quality forage. As such, we do not anticipate that the DEG identified in this study were reflective of cows consuming different rations.

Apart from the diet, hormone shifts associated with the transition from the dry period to lactation can significantly affect bovine MG (Hurley 1989), and these transformations continue to occur throughout lactation as evidenced by the effect of lactation stage on milk production (McManaman and Neville 2003). During the dry period, the MG is believed to regenerate in preparation for the next lactation cycle (Collier et al. 2012). Therefore, it is important to explore the complex molecular mechanisms underlying the transition to lactation. Here, we have detected 881 DEG in the MG of lactating, compared with dry cows. Of these DEG, 605 were upregulated and 276 were downregulated. Among the 881 DEG, a total of 25 upregulated DEG primarily related to various protein metabolism (including AA transporters: SLC38A3, SLC7A5, SLC7A8; AA metabolism: SARS, PAH, ASNS, GCAT; ribosomal proteins: RPL22, RPS3A, RPS27A, RPL39; and caseins: CSN1S2 and CSN3), lipid metabolism (GPD1, ACSS1, FABP3, FASN, HADHB, SLC27A6), energy metabolism (FBP1, ACSS1, PPA1, HK1), and glucose transport (SLC2A4 and SLC1A2). Only two downregulated DEG (EIF4EBP1 and SESN1) were found in consistent expression patterns with their corresponding DEP in our 2D-proteomic study (Dai et al. 2017b). This is not surprising because a series of studies have found that it is not uncommon for transcriptome data to be inconsistent with the corresponding proteome data due to potential post-modifications or the different identification methods of differential expression patterns (Ghazalpour et al. 2011, Goldberg and Brunengraber 1980, Nagaraj et al. 2011). When the DEG in our study were compared to other transcriptomic efforts, half of the 881 DEG overlapped with DEG reported in previous studies. The DEG which did not overlap with previous studies could be because the studies used different species of cows (American Holstein vs. China Holstein), different physiological stages (early, peak, or late lactation in other studies vs. only mid-lactation in this study), different diets (diet supplemented with other forages or some fatty acids vs. diet supplemented with alfalfa as forage), or different methods of sampling tissues (biopsy in other studies vs. slaughter in this study). For example, a recent study from Seo et al. (2016) on the MG from American Holstein during different stages of lactation by RNA-seq found that 271 DEG were characterized as the milk production-related genes, among which 54 DEG mainly involved in lipid metabolism, protein metabolism, mammary gland development, and defense response were in line with the expression patterns of those DEG in this study. In contrast, apart from lingual antimicrobial peptide (LAP), the other top 4 DEG (LALAB, CSN1S1, FASN, and CXCL2) in this study did not agree with those 4 top DEG detected in Seo et al.'s study—mammary serum amyloid A3.2 (M-SAA3.2),

Table 1 KEGG pathway enrichment of the differentially expressed genes in the mammary gland of dairy cows during lactation versus non-lactation

KEGG pathway	Upregulated gene no.	Upregulated gene symbol	Downregulated gene no.	Downregulated gene symbol	*P* value of Fisher' exact test	−Log*P* value
Metabolic pathways	28	IDI1;PEMT;PYCR1;DHCR24;LALBA;HPSE; HADHB;SLC27A6;SPTLC3;ACSS1;SULF2; SARS;ATP2C2;PMM2;GPAM;AGPAT6; ALDH3B2;SCLY;ASNS;GCAT;PAH; ALDH1L2;PSAT1;HPGD;FBP1;ATP13A4	4	HINT1;PFKFB3;MMP15; FLAD1;RDH16	0.02	1.62
Protein digestion and absorption	10	SLC7A4;SLC1A5;KCNMA1;KCNK6; KCNJ15;ATP8B4;ATP2C2; SLC7A8;SLC1A5;COL12A1	0		0.00	2.88
Ribosome	4	RPS27A;RPL22;RPS3A;RPL39	0		0.01	2.02
Aminoacyl-tRNA biosynthesis	2	SARS;ASNS	0		0.01	2.01
mTOR signaling pathway	0		3	DDIT4;SESN1;EIF4EBP1	0.03	1.48
Glycine, serine and threonine metabolism	4	PSAT1;SARS;GCAT;GATM	0		0.01	1.87
PPAR signaling pathway	7	SCD;ADIPOQ;LPL;SLC27A6;FABP3; PLIN;GK	0		0.00	2.33
Insulin signaling pathway	7	HK1;INSR;IRS1;INSIG1;SLC2A4; ACACA;FBP1	0		0.00	2.42
Glycolysis / Gluconeogenesis	12	HK1;ACSS1;GALM;GCK;GPD1;FBP1; GK;PCK2;ATF3;GPAM; AGPAT6; ACACA	0		0.04	1.45
Oxidative phosphorylation	3	ND6;PPA1;COA1	0		0.03	1.46
MAPK signaling pathway	4	IKBKE;FOS;RASGEF1C;NR4A1	4	IL1B;CD14;FGF2;FGFBP2	0.45	0.34
p53 signaling pathway	0		4	CDKN1A;SESN1;GADD45G; CD82;	0.02	1.60
TGF-beta signaling pathway	0		2	TGFB1;TGFB2	0.05	1.31
Chemokine signaling pathway	0		9	CXCR2;CXCL2;CXCL2;CXCL2; CXCL10;CCL17;CCL2; CCL20;CCR1	0.01	2.07
Cytokine-cytokine receptor interaction	0		13	IL1B;CSF3R;IL-8; IL33;CXCR2; CXCL2; CXCL2;CXCL2; CXCL10; CCL17;CCL2;CCL20; CCR1	0.01	2.19
Ribosome	4	RPS27A;RPL22;RPS3A;RPL39	0		0.01	2.02

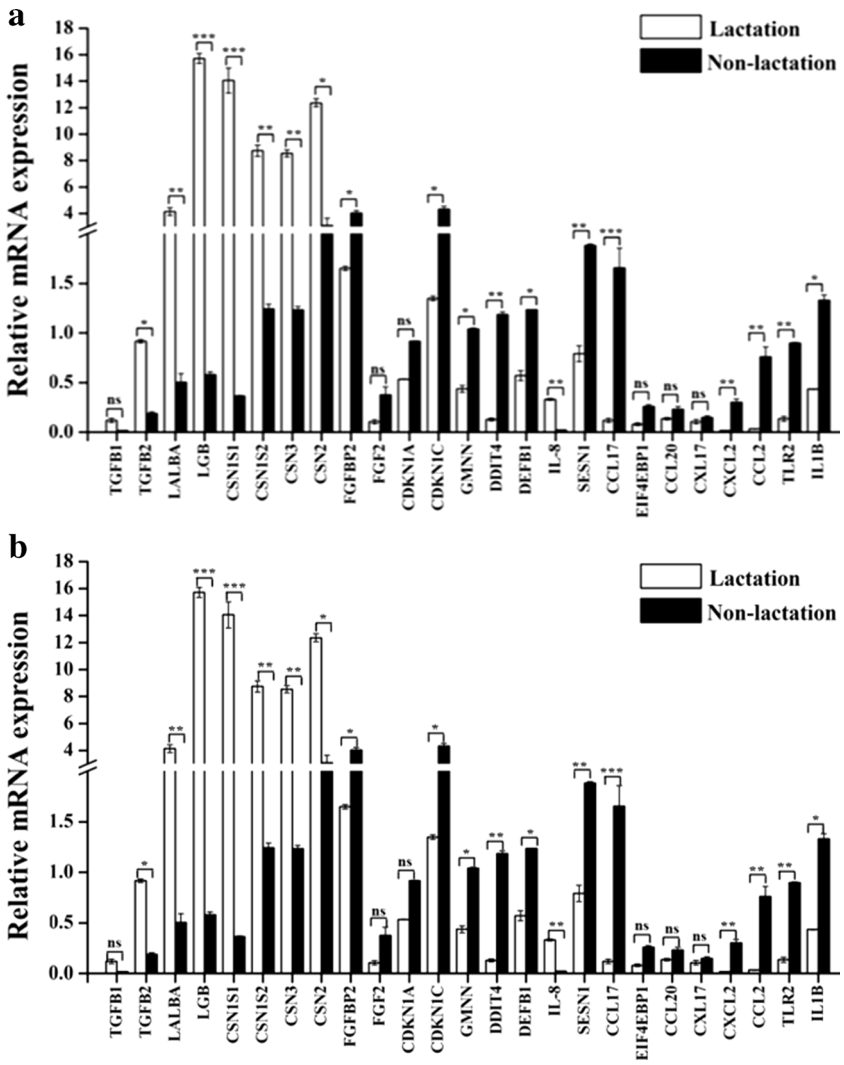

Fig. 4 Real-time PCR analysis of mRNA expression changes of genes involved in nutrient metabolism (**a**) caseins and mammary health (**b**) in the mammary gland of dairy cows during lactation versus non-lactation. Relative mRNA expression levels were normalized by the levels of β-actin and RPS9. Error bars represent the standard deviation. The symbols ***, **, and * indicate that the difference in gene expression between the two groups reached $P < 0.001$, $P < 0.01$, and $0.01 < P < 0.05$, respectively

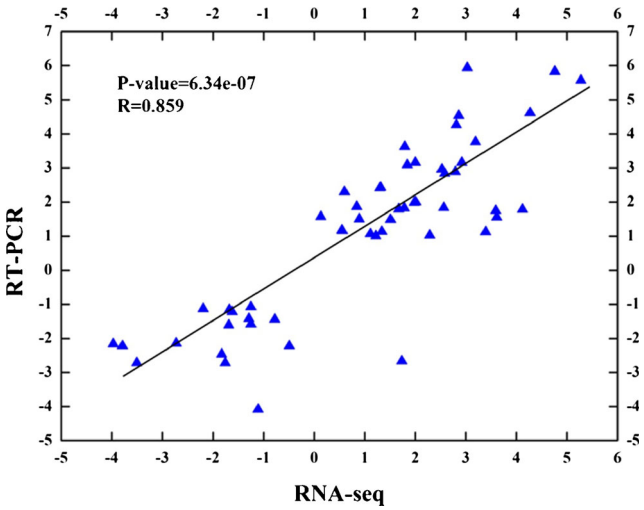

Fig. 5 Correlation of mRNA expression level of 51 differentially expressed genes in the mammary gland of dairy cows during lactation versus non-lactation using RNA-Seq and RT-PCR

sarcosine dehydrogenase (SARDH), nitric oxide synthase 3 (NOS3), and claudin 6 (CLDN6). Also, a study detected several DEG encoding FABP3, FASN, SCD, PLIN2, whey proteins (LALBA and LGB) and caseins (CSN1S1, CSN1S2, CSN2 and CSN3) upregulated significantly in MG of lactating, compared with the non-lactating dairy goats (Shi et al. 2015), which aligned well with the present study which found these corresponding DEG detected in cattle. Overall, the coordinated responses identified in this study generally agreed directionally with those identified in previous studies comparing lactating and dry mammals.

The 51 DEG selected for RT-PCR verification reflected three criteria: the expression patterns of these DEG agreed with the expression patterns of those corresponding DEG or DEP found in the previous studies and our 2D-proteomic study; the 51 DEG were the most highly up-/downregulated genes found in this study; and the 51 DEG were most enriched in the milk-production-related processes. Of these genes, 4 genes (DNAJC12, SPC3, ASNS, DDIT4, and JUNB) had

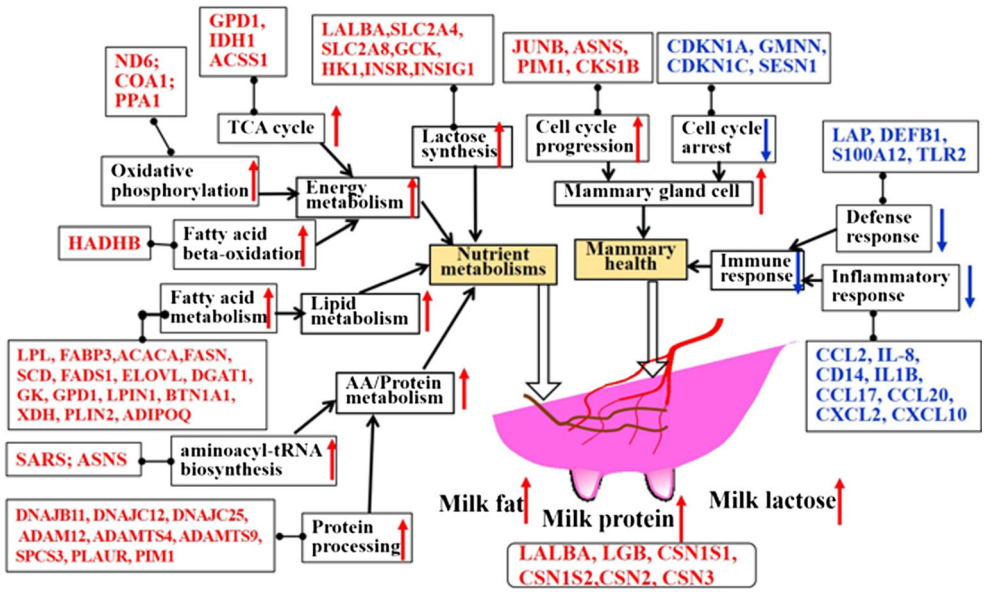

Fig. 6 The whole view of the regulated units associated with molecular mechanisms of milk production in the mammary gland of dairy cows underlying lactation. Color coding is as follows for items from the present study: red letters represent the upregulated genes, blue letters a represent upregulated the downregulated genes. The red arrows indicated enhanced processes; the blue arrows indicated lower processes

not previously been identified as significantly expressed in MG of lactating dairy cows compared to the non-lactating cows.

Energy metabolism

Milk production, especially milk protein synthesis, is an energetically costly process (Hanigan et al. 2009). In ruminants, the activation of carbohydrate metabolism and subsequent ATP synthesis and glucose metabolism is crucial to satisfy the ATP requirement for milk production and milk protein synthesis. The differentially expressed energy metabolism-related pathways in this study suggested enhanced activity of glycolysis/gluconeogenesis (FBP1, DDIT4), the TCA cycle (ACSS1, IDH1), and the pentose phosphate pathway (HIBADH). The enhanced expression of fructose-1,6-bisphosphatase 1 (FBP1; FC = 2.21) and the reduced expression of DNA-damage-inducible transcript 4 (DDIT4; FC = 0.46) support enhanced glycolysis activity. Moreover, the upregulated gene acetyl−CoA synthetase 1 (ACSS1; FC = 2.94) linking acetyl−CoA synthesis into tricarboxylic acid (TCA) cycle may function to enhance substrate procurement by the TCA cycle. The higher abundance of FBP1 and ACSS1 in the bovine lactating MG was consistent with transcript upregulation at the onset of lactation and upregulated gene expression during lactation (Bionaz and Loor 2011; Finucane et al. 2008) and the corresponding 2.9-fold upregulation of FBP1 protein and 2.2-fold upregulation of ACSS1 protein in the MG of lactating cows compared with the dry cows (Dai et al. 2017b). Additionally, the 6.78-fold upregulation of HIBADH gene identified in this study was in line with the 8.66-fold upregulation of its corresponding protein in lactating group compared to the dry group (Dai et al. 2017b). Consistent with its high abundance in protein expression (36.24-fold) (Dai et al. 2017b), the upregulated DEG idehydrogenase 1 (IDH1; FC = 15.42) would largely promote isocitrate conversion into oxalosuccinate and NADH production in the TCA cycle further for energy generation (Rawson et al. 2012). Also, we detected four upregulated glucose transporter genes SLC1A2 (FC = 3.96), SLC2A3 (FC = 2.23), SLC2A4 (FC = 15.54), and SLC2A8 (FC = 8.94), among which the other three glucose transporter genes except SLC2A4 were all found upregulated in bovine MG during lactation versus the dry period (Bionaz and Loor 2011). Also, the corresponding proteins both of GLUT4 and GLUT2 were found upregulated in the lactating group versus the dry group (Dai et al. 2017b). Then, in the lactation group, the higher abundance of three mitochondrial enzymes involved in mitochondrial membrane respiratory chain [NADH-ubiquinone oxidoreductase chain 6 (ND6), FC = 19.40; cytochrome c oxidase assembly 1 (COA1), FC = 3.31; pyrophosphatase 1 (PPA1), FC = 2.19] contributed to support elevated ATP demand through mitochondrial electron transport and oxidative phosphorylation. Additionally, the enhanced abundance of PPA1 in this study agreed with its 6.39-fold upregulation of PPA1 protein in lactating MG versus the non-lactating MG within our previous proteomic study (Dai et al. 2017b), and this was the first time to detect mammary PPA1 gene was more highly expressed in the MG lactating dairy cows compared to dry cows. These genes likely coordinate to ensure sufficient energy availability for milk production and mammary tissue maintenance in lactating cows. Indeed, it was shown that the machinery associated with energy generation was positively regulated in the lactating bovine MG during lactation, suggesting the MG enhances glycolysis and the TCA cycle but also mitochondrial electron transport and oxidative phosphorylation.

Lipid metabolism

Most milk FA are synthesized de novo when sufficient fatty acid is absorbed from blood into the MG. Within the lactation group versus the dry group, the expression of 24 genes involved in various mammary FA metabolisms was of increased abundance. These included the following: lipoprotein lipase (LPL), intracellular FA trafficking (FABP3, SLC22A16)), short-chain intracellular FA activation (ACSS1, ACSS2), de novo FA synthesis (ACACA, FASN), desaturation (SCD5, DGAT1, FADS1), triacylglycerol (TAG) synthesis (GK, GPD1, DHCR24, COQ2, AGPAT6, GPAM, LPIN1), lipid droplet formation (BTN1A1, XDH, PLIN2), and fatty acid beta-oxidation (ADIPOQ, HADHB). The change in FA uptake capacity by the MG was expected given the typical breakdown of body fat stores that occurs to avoid negative energy balance in lactating dairy cows. Compared with a 2D-proteomic study of Dai et al. (2017b), 6 DEG (including GPD1, ACSS1, FABP3, FASN, HADHB, SLC27A6) shared the consistent upregulation expression patterns with their corresponding proteins in lactating, compared to the dry groups. Besides, almost 80% of the above milk fat-related DEG were in the same expression patterns with these DEG found in the similar previous studies (Bionaz and Loor 2008, Finucane et al. 2008, Seo et al. 2016).

Mammary cells usually take up long-chain FA from lipoproteins and albumin-bound FA. Very low-density lipoprotein or chylomicrons are anchored to mammary endothelium by lipoprotein lipase (LPL), which then hydrolyzes TAG in the lipoprotein core to release FA (Fielding and Frayn 1998). The observed LPL upregulation (FC = 4.22) at mid-lactation was also found as early as the onset of milk synthesis (Finucane et al. 2008). Fatty acid binding protein (FABP) can bind long chain FA and their CoA thioesters, but not medium or short chain FA in MG of ruminants (Whetstone et al. 1986). Here, the highly upregulated FABP3 (FC = 24.60) was in line with the large cytosolic content of its protein in mammary epithelial cells (Whetstone et al. 1986) and its corresponding upregulated DEP in a proteomic study (Dai et al. 2017b). In addition, FABP3 could protect mammary cells from negative effects of activated FA via binding of activated acyl-CoA and prevent inhibition of acetyl-CoA carboxylase alpha (ACACA, the rate-limiting step in denovo FA synthesis for short chain FA production) and of stearoyl-CoA desaturase 5 (SCD5, essential for saturation of milk fat) [39]. As a result, upregulation of FABP3 may promote the supply of FA for SCD or other enzymes involved in TAG synthesis in the lactating bovine MG. Two bovine acetyl-CoA synthetase (ACSS) isoforms have been characterized in MG—ACSS1 primarily found in mitochondria and ACSS2 mainly existing in cytosol (Fujino et al. 2001). The lower abundance of ACSS1 (FC = 2.94) compared with ACSS2 (FC = 20.27) throughout stages of lactation is in agreement with the reduction of acetate use and relatively high lipid synthesis (Bauman and Griinari 2003). Additionally, the cytosolic fatty acid synthase (FASN) would promote generation of palmitate and short-chain FA (Bionaz and Loor 2008). Here, the upregulation of FASN (FC = 37.72) during lactation was consistent with previous studies identifying its increased expression from pregnancy through lactation (Bionaz and Loor 2008), and the 19-fold upregulation of its corresponding proteins in our 2D-proteomic study (Dai et al. 2017b). All these genes involved in TAG synthesis were substantially upregulated when dairy cows were in mid-lactation, suggesting enhanced milk fat synthesis during this period. Also, two upregulated ADIPOQ (adiponectin; FC = 4.48) and HADHB (hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, beta subunit; FC = 3.48) involved in fatty acid β-oxidation may further help supply energy for lactation. Here, the enhanced abundance of ADIPOQ agreed with a previous study identifying its increase during lactation (Bionaz and Loor 2008). The upregulated HADHB was consistent with its corresponding upregulated protein in the proteomic study (Dai et al. 2017b).

Dynamic amino acid and protein metabolism

As with other tissues, sufficient AA availability within the MG is required for protein synthesis in dairy cows. The synthesis processes of milk protein include transcription, translation, and dynamic AA metabolism. Differential expression of 25 genes associated with mammary AA uptake from blood (upregulated: SLC1A2, SLC1A5, SLC7A4, SLC7A8, SLC38A3), intracellular AA metabolism (upregulated: SARS, ASNS, PYCR1, PAH, PSAT1, GCAT), transcription (upregulated: JUNB, FOS, ATF3, LPIN1, ESRRA; downregulated: GMNN, TFCP2L1, BARX2), and translation (upregulated: RPS27A, RPL39, RPL22, RPS3A; downregulated: SESN1, EIF4EBP1, DDIT4) were identified during lactation. Comparing this study to the expression patterns in a proteomic study of Dai et al. (2017b), three of the above five corresponding AA transporter proteins (SLC38A3, SLC7A5, SLC7A8), all the four ribosomal proteins (RPL22, RPS3A, RPS27A, RPL39), four upregulated proteins (SARS, PAH, ASNS, GCAT) involved in intracellular AA metabolism, and the two downregulated protein EIF4EBP1 and SESN1 were consistent in expression. Additionally, a total of seven DEG (including SLC1A5, SLC7A2; RPL22, RPL39; GCAT; EIF4EBP1) involved in various AA/protein metabolisms previously identified during lactation versus non-lactation (Bionaz and Loor 2011) were consistent with this study.

Sufficient AA supply to the MG is important for regulation of translation, and AA transport into the MG appears to be one of the major limitations for milk protein synthesis (Bionaz and Loor 2011). In support of this idea, we identified several AA transporters in higher abundance: L-type AA transporter SLC7A8 (FC = 2.86), sodium-coupled neutral AA transporter

SLC38A3 (FC = 8.50), sodium-dependent neutral AA transporter SLC1A5 (FC = 3.54), anionic AA transporter SLC1A2 (FC = 3.96), and cationic AA transporter SLC7A4 (FC = 2.81). With the exception of SLC7A4, all these upregulated transporters in this study have been also found in higher expression at mid-lactation (Baik et al. 2009, 2011). With sufficient AA absorption stimulated by these upregulated AA transporters, the subsequent intracellular AA metabolism is a highly dynamic process in lactating mammals. For example, two DEG, seryl-tRNA synthetase (SARS; FC = 12.38) and asparagine synthetase (ASNS; FC = 3.47), were involved in the synthesis of seryl-tRNA and aspartyl-tRNA, respectively. These two corresponding DEP were also found upregulated 5.88-fold (SARS) and 8.44-fold (ASNS) in the 2D proteomic study from Dai et al. (2017b); while the Bionaz work found another DEG—Leucine tRNA-synthetase (LARS) was approximately threefold upregulated (Bionaz and Loor 2011). The difference in tRNA-synthease identified between these studies may result from the different species of cows used and different lactation periods selected for MG tissue collection. Differential expression of pyroline-5-carboxylate reductase 1 (PYCR1; FC = 5.37) and phosphoserine aminotransferase 1 (PSAT1; FC = 3.10) was also identified, which are related to synthesis of L-proline and L-serine, respectively. The enzymes phenylalanine hydroxylase (PAH; FC = 3.58) and glycine C-acetyltransferase (GCAT; FC = 2.85), associated with the catabolism of L-Phe and L-Thr, respectively, were also upregulated. A total of the four DEG (SARS, ASNS, PAH, and PYCR1) were shown, to some degree, to be upregulated in the lactating bovine MG (Ouattara et al. 2016). The upregulation of these genes supports the importance of intracellular AA metabolism during lactation. Also, a total of 22 DEG (13 upregulated and 9 downregulated) involved in transcription and 7 DEG (6 upregulated and 1 downregulated) related to translation were identified. As expected, 4 ribosomal proteins (RPS27A, RPS3A, RPL39, and RPL22) were highly expressed during lactation, compared with the dry period, all of which were found in the same expression patterns of their corresponding DEP in our proteomic study (Dai et al. 2017b). Surprisingly, no initiation, elongation, or termination factors most commonly involved in protein translation were differentially expressed in this study. We did find that a crucial end-of-pathway gene for mTOR signaling (eukaryotic translation initiation factor 4E binding protein 1, EIF4EBP1; FC = 0.33) was downregulated in the lactating cows compared with the dry cows, which is consistent with the findings of the proteomic study from Dai et al. (2017b) and the work of Bionaz and Loor (2011). In all, several DEG (GMNN, FOS, JUNB, and DDIT4) related to transcription and translation were newly identified in lactating MG versus non-lactating MG of dairy cows, but these genes have been identified as milk-production-related genes in humans and mice (Clarkson et al. 2004, Katiyar et al. 2012, Stein et al. 2009).

Protein folding, post-translational modification, and protein transport

Protein folding and modification are essential to convert newly synthesized proteins into biologically functional forms (Englander and Mayne 2014), which also dramatically expands the functional diversity of proteins (Bah and Forman-Kay 2016). We detected that during lactation, a series of genes associated with protein folding (DNAJB11, DNAJC12, DNAJC25), protein processing (ADAM12, ADAMTS4, ADAMTS9, ADAMTS2, SPCS3), and protein modification (PLAUR, PIM1, GCK, PIK3C2G, GK, ASB11, SOCS3) were of upregulated expression. Additionally, genes responsible for intracellular protein transport (RHOF, RASD1, RASEF, WIPI1, RAB7B, SEC24D, RAB26, CNST, PLEK) and all six kinds of milk proteins (LALBA, LGB, CSN1S1, CSN1S2, CSN3, CSN2) excreted from the plasma membrane were all enhanced during lactation. Because of limitations in the 2D-proteomic approach (Dai et al. 2017b), DNAJB11, SEC24D, and two caseins (CSN1S2 and CSN3) were found in higher abundance, which was in line with their upregulated DEG here. The other DEG involved in protein folding, post-translational modification and protein transport above were not identified in our proteomic study. In contrast, all of the four caseins and the two whey proteins (LALBA, LGB) were also found in enhanced abundance in MG of lactating dairy cows (Seo et al. 2016) and lactating goats (Shi et al. 2015). Previous studies have not identified DNAJC12 (FC = 5.4) and SPC3 (FC = 2.2) as differentially expressed in the MG of lactating cows.

Protein processing typically occurs in the ER or Golgi and is the process by which proteins acquire modifications that allow them to be functionally active (Krieg et al. 1989). We identified four DEG related to proteolysis, three enzymes from ADAM metallopeptidase with thrombospondin type 1 motif (ADAMTS) family (including ADAMTS2, FC = 2.05; ADAMTS4, FC = 8.54; ADAMTS9, FC = 2.17), and ADAM12 (FC = 2.77); the three of which except ADAMTS2 were in higher abundance of lactating MG from dairy cows fed alfalfa-based diet versus those fed corn stover-based diet (Dai et al. submitted to BMC Genomics). These products play essential roles in post-translational processing of procollagen molecules, the precursors to form type I and type III collagens (Kesteloot et al. 2007). Thus, the increased abundance of these three ADAM enzymes may indicate enhanced extracellular matrix (ECM) growth and further stimulate mammary cell growth. Additionally, three genes (DNAJB11, FC = 2.08; DNAJC12, FC = 5.40; and DNAJC25, FC = 2.41) related to protein folding located to ER were in higher abundance. As an ER molecular chaperone, DnaJ homolog subfamily B member 11 (DNAJB11; also known as ERj3) binds to BiP, a major molecular chaperone involved in ER-associated degradation (ERAD). This

complex then aggregates heat shock protein 70 (Hsp70) ATPase to stimulate ERAD (Qiu et al. 2006). The co-chaperone of Hsp70 (DNAJC12) and a member of Hsp40 (DNAJC25) were separately related to ER stress (Choi et al. 2014) and chaperoning substrate specificity (Heldens et al. 2010). Additionally, DNAJC12, DNAJB11, and DNAJC25 were also found in enhanced expression level in lactating bovine MG fed high-quality forage compared to those fed low-quality forage (Dai et al. 2017a). In summary, these three major co-chaperone genes may combine to ensure the correct protein folding and normal function of various intracellular proteins in lactating cows (Rawson et al. 2012).

A total of five upregulated DEG were involved in protein phosphorylation. Of these, the PIM1 oncogene has diverse biological roles in cell survival, proliferation, and differentiation through modulating PRAS40 (proline-rich Akt substrate of 40 kDa) phosphorylation at Thr^{246} and AKT phosphorylation site, which subsequently affects mTOR activity (Zhang et al. 2009). Additionally, SOCS3 (the suppressor of cytokine signaling 3, a gene accelerating ubiquitin-dependent degradation) were downregulated, which was identified in reduced abundance in lactating MG of mouse than the non-lactating group (Rui et al. 2002). Downregulation of SOCS3 (FC = 0.38) may help inhibit progression of ubiquitin-dependent degradation and increase the net protein synthesis in lactating MG. In summary, the above DEG work together to promote maximal protein phosphorylation and reduce protein degradation within the lactating MG.

Also, nine upregulated DEG involved in protein transport were identified during lactation, including three DEG (RHOF, RASD1, RASEF) related to intracellular protein transport, three DEG (WIPI1, RAB7, SEC24D) that mediate the transport of Glogi targeting from other cellular compartments, and three DEG (RAB26, CNST, PLEK) that facilitate protein transport from Golgi to plasma membrane. These identified DEG are in line with the expected increase in milk protein synthesis during lactation. Among the nine protein-transport-related DEG, five genes (RAB7, SEC24D, RAB26, RASD1, PLEK) were determined as milk production-related genes in lactating Holstein MG compared to the non-lactating MG (Seo et al. 2016).

Regulation of mammary cell number and mammary development

Mammary cell proliferation is an important biological mechanism supporting lactation. Much like adipocytes (Taga et al. 2012), mammary cell proliferation is determined by the progression of cell cycle precursors and the prevention of cell cycle arrest. Our functional analysis showed that many DEG were directly or indirectly (through effective interactions) involved in upregulation of cell cycle progression (JUNB, ASNS, PIM1, CKS1B) and downregulation of cell cycle arrest (CDKN1A, CDKN1C, GMNN, SESN1). Interestingly, this is the first time that ASNS and JUNB were identified as helpers in promoting the bovine mammary cell cycle, and as a result, the exact mechanisms of action are unknown. In contrast, the downregulation of cyclin-dependent kinase inhibitor 1A (CDKN1A; FC = 0.44) and its partner CDKN1C (FC = 0.45) suggests mediation of cell cycle arrest in response to DNA damage not only by inactivating G1-phase cyclins complexes but also through other processes, which possibly include direct interaction with proliferating cell nuclear antigen to inhibit DNA replication, and indirect effects mediated by interaction with other cell cycle regulators (Cazzalini et al. 2010). Intriguingly, SESN1 has been previously determined as the negative upstream regulator of mTORC1 signaling pathway in human 293T cell (Chantranupong et al. 2014), but here SESN1 was found to be related to the mammary cell cycle. In light of this previous functional identification, the role of SESN1 in the mammary cell cycle is unclear. In contrast, the 2 downregulated cyclin-dependent kinase inhibitors (CDKN1A and CDKN1C) in this study were detected as having a consistent expression pattern with those in MG of mouse from lactation through involution (Stein et al. 2009).

Additionally, we found a number of DEG related to mammary cell growth process, including two upregulated collagens (COL8A1, COL1A2) involved in epithelial cell proliferation, four upregulated epithelial cell development-related genes (PTHLH, DGAT1, RAB26 and AGPAT6), two downregulated transforming growth factors (TGFB1, TGFB2), and two upregulated fibroblast growth factor members (FGFBP2 and FGF2). Among these, parathyroid hormone-like hormone (PTHLH) is crucial for regulating the transition from budding to branching in the MG of mice (Juppner et al. 1991). Here, the higher abundance of PTHLH (FC = 12.25) may suggest that the process of mammary differentiation in the lactating MG was stimulated, which has been identified at the onset of lactation in previous studies (Finucane et al. 2008). Comparatively, transforming growth factor 1 (TGFB1) and 2 (TGFB2) inhibited functional differentiation and lactogenesis in mammary explants from pregnant mice (Robinson et al. 1991). Meanwhile, fibroblast growth factor 2 (FGF2) combined with its binding gene fibroblast growth factor binding protein 2 (FGFBP2) may function in the development and reorganization of fibroblast and mammary gland tissue (Wang et al. 2008). Compared to the previous studies of the lactating Holstein MG, two DEG (FGFBP2 and PTHLH) were in consistent expression patterns with those of this work (Seo et al. 2016). Additionally, COL8A1 was identified to be upregulated in the work of Li et al. (2016). Thus, together with the downregulation of TGFB1 (FC = 0.04) and TGFB2 (FC = 0.49), the upregulation of FGFBP2 (FC = 2.68) and FGF2 (FC = 5.50) may help ensure optimal mammary cell growth and maximum mammary cell differentiation. To our

knowledge, the role for AGPAT6 in mammary epithelial structure is unclear.

We also identified four upregulated DEG (DHCR24, ASNS, THBS1, SOCS3) involved in negative regulation of apoptosis, and five downregulated DEG (AGT, CRYAB, DDIT4, SFRP2, ITPR1) involved in positive regulation of extrinsic/intrinsic apoptotic process, and one upregulated DEG (MFGE8) related to positive regulation of apoptotic cell clearance. Collectively, all these changes suggest that regulation of mammary cell precursor proliferation by controlling cell cycle progression and apoptosis or by delaying cell cycle arrest is an important process in mammary gland growth and development during lactation.

Lower immune-response-related processes

The MG of dairy cows, which is prone to infection by various bacteria, requires local and systemic immune defenses to cope with various pathogens (Sordillo et al. 1997). Thus, the activated immune processes can act as a marker of the health status of the bovine MG to avoid every potential infection. In this study, we identified a set of DEG of lower abundance involved in defense responses (LAP, DEFB1, S100A12, TLR2, FGR, SERPINE1), inflammatory response (CCL2, IL-8, CD14), immune responses (CCL17, CCL20, CXCL2, IL1B, PRG3, CFB), and immunoglobulin secretion (IL33). Meanwhile, three genes (HSPA5, ATF3 and PYCR1) associated with response to stress were of higher expression.

The top downregulated DEG involved in defense responses were beta-defensin 1 (DEFB1;FC = 0.16) and toll-like receptor 2 (TLR2; FC = 0.23); both of which had much close relationship with bovine MG responses to resist disease (Gunther et al. 2009, Moyes et al. 2010). When the lactating bovine MG was challenged with *Escherichia coli*, the expression of DEFB1 was sharply upregulated (Gunther et al. 2009), which agrees with the downregulation of DFB1 in this study. Also, the lower inflammatory responses are indicated by the reduced mammary cellular response to LPS, which involves downregulation of the chemokine CCL2 (FC = 0.15), IL8 (FC = 0.06) and the cell surface protein CD14 (FC = 0.45). In addition, the reducing in abundance of LAP (FC = 0.13) and CCL2 (FC = 0.15) of reduced abundance was in line with their lower levels (LAP FC = 0.25; CCL2 FC = 0.09) in MG of lactating Holstein (Seo et al. 2016). In summary, the downregulation of these genes related to immune response may suggest that the lactating dairy cows have lower possibility to infect diseases and higher access to health than the dry cows.

Among the 7 DEG related to immune response, 3 cytokines CCL17, CCL20, CXCL2, and IL1B were the top 4 downregulated genes, which were previously identified to be closely related to mastitis in dairy cows (Kerr and Wellnitz 2003, Swanson et al. 2009). Cytokines represent a numerous and diverse group of soluble factors that modulate important mammary leukocyte and endothelial cell populations (Sordillo 2005). The above four cytokines have been studied in bovine mammary immunology, and IL1B is the most extensively characterized related mammary mastitis (Gunther et al. 2009, Moyes et al. 2010, Sordillo et al. 1997). The onset of milk synthesis and secretion is accompanied by a high energy demand, and an increased oxygen requirement (Sordillo 2005). Previous work has related Hsp70 member 5 (HSPA5) and ATF3 to induced ER stress pathway changes during the transition from pregnancy to lactation in dairy cows (Invernizzi et al. 2012). The gene PYCR1 is related to heat stress responses in dairy cows (Zheng et al. 2014). The three genes in higher abundance may reflect the MG of lactating cows encountered a lot of stresses from environment or the body itself. The role of immune status in the transition from lactation to the dry period has also been supported by previous studies. For example, the downregulation identified in CXCL2 and IL1B was also identified in the work (Seo et al. 2016).

Conclusions

This transcriptomic investigation provides prospective into the cellular and molecular-level features of the lactating bovine MG. Our data suggest two major mechanisms (nutrient metabolism and health of the MG) contributing to maintaining lactation in dairy cows. These functions can be broken down into the following: (i) enhanced mammary growth/development through lower expression of cell cycle arrest-related genes and cell apoptosis-related genes, and higher expression of cell cycle progression-related genes and cell growth-related genes; (ii) increased energy generation through higher expression of energy metabolism-related genes, fatty acid β-oxidation-involved genes, and the final oxidative phosphorylation-associated genes; (iii) enhanced milk component (including milk lactose, lipid and protein) production through higher expression of glucose/fatty acid/amino acid transporter genes, genes involved in macromolecule metabolisms (including glucose/amino acid/lipid/ protein metabolisms), and genes related to protein folding/modification/transport and lipid excretion; and (iv) lower immune system response through reduced expression of genes associated with defense response, immune response, inflammatory response, and immunoglobulin secretion. These mechanisms show the adaptations of the bovine MG to lactation as compared with the dry period. Among the most interesting findings of our study is the identification of genes expressed during lactation stage MG growth that may act as regulators of mammary cell proliferation by controlling cell cycle progression and apoptosis or by delaying cell cycle arrest. These new findings are of interest because adequate development of mammary tissue is a major step in promoting metabolic adaptations, both at birth for neonate survival and in adult life for productive efficiency.

This transcriptomic analysis of the bovine MG provides more views into mammary tissue functional responses during the shift between lactation and the dry period.

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Compliance with ethical standards The procedures of this study were approved by the Animal Care and Use Committee of Zhejiang University (Hangzhou, China) and were in accordance with the university's guidelines for animal research.

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

Authorship W.T. Dai and H.Y. Liu designed the study; W.T. Dai mainly performed the research and Y.X. Zou helped the PCR experiment; W.T. Dai analyzed the whole data and wrote the paper; R.R. White helped revise the manuscript.

References

Bah A, Forman-Kay JD (2016) Modulation of intrinsically disordered protein function by post-translational modifications. J Biol Chem 291(13):6696–6705. https://doi.org/10.1074/jbc.R115.695056

Baik M, Etchebarne BE, Bong J, VandeHaar MJ (2009) Gene expression profiling of liver and mammary tissues of lactating dairy cows. Asian Austral J Anim 22(6):871–884. https://doi.org/10.5713/ajas.2009.90061

Bauman DE, Griinari JM (2003) Nutritional regulation of milk fat synthesis. Annu Rev Nutr 23(1):203–227. https://doi.org/10.1146/annurev.nutr.23.011702.073408

Bellmann O (1976) Hormonal-regulation of lactation. Klin Padiatr 188(5):385–395

Bionaz M, Loor JJ (2008) Gene networks driving bovine milk fat synthesis during the lactation cycle. BMC Genomics 9(1):366. https://doi.org/10.1186/1471-2164-9-366

Bionaz M, Loor JJ (2011) Gene networks driving bovine mammary protein synthesis during the lactation cycle. Bioinf Biol Insights 5:83–98. https://doi.org/10.4137/bbi.s7003

Cazzalini O, Scovassi AI, Savio M, Stivala LA, Prosperi E (2010) Multiple roles of the cell cycle inhibitor p21CDKN1A in the DNA damage response. Mutat Res-Rev Mutat 704(1-3):12–20. https://doi.org/10.1016/j.mrrev.2010.01.009

Chantranupong L, Wolfson RL, Orozco JM, Saxton RA, Scaria SM, Bar-Peled L, Spooner E, Isasa M, Gygi SP, Sabatini DM (2014) The Sestrins interact with GATOR2 to negatively regulate the amino-acid-sensing pathway upstream of mTORC1. Cell Rep 9(1):1–8. https://doi.org/10.1016/j.celrep.2014.09.014

Choi J, Djebbar S, Fournier A, Labrie C (2014) The co-chaperone DNAJC12 binds to Hsc70 and is upregulated by endoplasmic reticulum stress. Cell Stress Chaperones 19(3):439–446. https://doi.org/10.1007/s12192-013-0471-6

Clarkson RW, Wayland MT, Lee J, Freeman T, Watson CJ (2004) Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. Breast Cancer Res 6(2):R92–109. https://doi.org/10.1186/bcr754

Collier RJ, Annen-Dawson EL, Pezeshki A (2012) Effects of continuous lactation and short dry periods on mammary function and animal health. Animal 6(03):403–414. https://doi.org/10.1017/S1751731111002461

Cui X, Hou Y, Yang S, Xie Y, Zhang S, Zhang Y, Zhang Q, Lu X, Liu GE, Sun D (2014) Transcriptional profiling of mammary gland in Holstein cows with extremely different milk protein and fat percentage using RNA sequencing. BMC Genomics 15:1

Dai W, Chen Q, Wang Q, White RR, Liu J, Liu H (2017a) Complementary transcriptomic and proteomic analyses reveal regulatory mechanisms of milk protein production in dairy cows consuming different forages. Sci Rep 7:44234. https://doi.org/10.1038/srep44234

Dai W, Wang Q, Zou Y, White RR, Liu J, Liu H (2017b) Short communication: comparative proteomic analysis of the lactating and nonlactating bovine mammary gland. J Dairy Sci 100(7):5928–5935. https://doi.org/10.3168/jds.2016-12366

Drackley JK (1999) ADSA foundation scholar award. Biology of dairy cows during the transition period: the final frontier? J Dairy Sci 82(11):2259–2273. https://doi.org/10.3168/jds.S0022-0302(99)75474-3

Englander SW, Mayne L (2014) The nature of protein folding pathways. Proc Natl Acad Sci U S A 111(45):15873–15880. https://doi.org/10.1073/pnas.1411798111

Fielding BA, Frayn KN (1998) Lipoprotein lipase and the disposition of dietary fatty acids. Brit J Nutr 80(06):495–502. https://doi.org/10.1017/S0007114598001585

Finucane KA, McFadden TB, Bond JP, Kennelly JJ, Zhao FQ (2008) Onset of lactation in the bovine mammary gland: gene expression profiling indicates a strong inhibition of gene expression in cell proliferation. Funct Integr Genomic 8(3):251–264. https://doi.org/10.1007/s10142-008-0074-y

Fujino T, Kondo J, Ishikawa M, Morikawa K, Yamamoto TT (2001) Acetyl-CoA synthetase 2, a mitochondrial matrix enzyme involved in the oxidation of acetate. J Biol Chem 276(14):11420–11426. https://doi.org/10.1074/jbc.M008782200

Ghazalpour A, Bennett B, Petyuk VA, Orozco L, Hagopian R, Mungrue IN, Farber CR, Sinsheimer J, Kang HM, Furlotte N, Park CC, Wen PZ, Brewer H, Weitz K, Camp DG 2nd, Pan C, Yordanova R, Neuhaus I, Tilford C, Siemers N, Gargalovic P, Eskin E, Kirchgessner T, Smith DJ, Smith RD, Lusis AJ (2011) Comparative analysis of proteome and transcriptome variation in mouse. PLoS Genet 7(6):e1001393. https://doi.org/10.1371/journal.pgen.1001393

Goldberg RP, Brunengraber H (1980) Contributions of cytosolic and mitochondrial acetyl-CoA syntheses to the activation of lipogenic acetate in rat liver. Adv Exp Med Biol 132:413–418

Gunther J, Koczan D, Yang W, Nurnberg G, Repsilber D, Schuberth HJ, Park Z, Maqbool N, Molenaar A, Seyfert HM (2009) Assessment of the immune capacity of mammary epithelial cells: comparison with mammary tissue after challenge with Escherichia Coli. Vet Res 40(4):31. https://doi.org/10.1051/vetres/2009014

Hanigan MD, France J, Mabjeesh SJ, McNabb WC, Bequette BJ (2009) High rates of mammary tissue protein turnover in lactating goats are energetically costly. J Nutr 139(6):1118–1127. https://doi.org/10.3945/jn.108.103002

Heldens L, Dirks RP, Hensen SM, Onnekink C, van Genesen ST, Rustenburg F, Lubsen NH (2010) Co-chaperones are limiting in a depleted chaperone network. Cell Mol Life Sci 67(23):4035–4048. https://doi.org/10.1007/s00018-010-0430-7

Hosseini A, Sharma R, Bionaz M, Loor JJ (2013) Transcriptomics comparisons of mac-T cells versus mammary tissue during late pregnancy and peak lactation. Adv Dairy Res 2013

Hurley WL (1989) Mammary gland function during involution. J Dairy Sci 72(6):1637–1646. https://doi.org/10.3168/jds.S0022-0302(89)79276-6

Ibeagha-Awemu EM, Li R, Ammah AA, Dudemaine P-L, Bissonnette N, Benchaar C, Zhao X (2016) Transcriptome adaptation of the bovine mammary gland to diets rich in unsaturated fatty acids shows greater impact of linseed oil over safflower oil on gene expression and metabolic pathways. BMC Genomics 17:1

Invernizzi G, Naeem A, Loor JJ (2012) Short communication: endoplasmic reticulum stress gene network expression in bovine mammary tissue during the lactation cycle1. J Dairy Sci 95:2562–2566. https://doi.org/10.3168/jds.2011-4806

Jiang Z, Zhou X, Li R, Michal JJ, Zhang S, Dodson MV, Zhang Z, Harland RM (2015) Whole transcriptome analysis with sequencing: methods, challenges and potential solutions. Cell Mol Life Sci 72(18):3425–3439. https://doi.org/10.1007/s00018-015-1934-y

Juppner H, Abou-Samra AB, Freeman M, Kong XF, Schipani E, Richards J, Kolakowski LF Jr, Hock J, Potts JT Jr, Kronenberg HM et al (1991) A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. Science (New York, NY) 254:1024–1026

Katiyar S, Jiao X, Addya S, Ertel A, Covarrubias Y, Rose V, Casimiro MC, Zhou J, Lisanti MP, Nasim T, Fortina P, Pestell RG (2012) Mammary gland selective excision of c-jun identifies its role in mRNA splicing. Cancer Res 72(4):1023–1034. https://doi.org/10.1158/0008-5472.can-11-3647

Kerr DE, Wellnitz O (2003) Mammary expression of new genes to combat mastitis. J Anim Sci 81(Suppl 3):38–47. https://doi.org/10.2527/2003.81suppl_338x

Kesteloot F, Desmouliere A, Leclercq I, Thiry M, Arrese JE, Prockop DJ, Lapiere CM, Nusgens BV, Colige A (2007) ADAM metallopeptidase with thrombospondin type 1 motif 2 inactivation reduces the extent and stability of carbon tetrachloride-induced hepatic fibrosis in mice. Hepatology (Baltimore, Md) 46:1620–1631. https://doi.org/10.1002/hep.21868

Krieg UC, Johnson AE, Walter P (1989) Protein translocation across the endoplasmic reticulum membrane: identification by photocross-linking of a 39-kD integral membrane glycoprotein as part of a putative translocation tunnel. J Cell Biol 109(5):2033–2043. https://doi.org/10.1083/jcb.109.5.2033

Kumar D, Bansal G, Narang A, Basak T, Abbas T, Dash D (2016) Integrating transcriptome and proteome profiling: strategies and applications. Proteomics 16(19):2533–2544. https://doi.org/10.1002/pmic.201600140

Li C, Cai W, Zhou C, Yin H, Zhang Z, Loor JJ, Sun D, Zhang Q, Liu J, Zhang S (2016) RNA-Seq reveals 10 novel promising candidate genes affecting milk protein concentration in the Chinese Holstein population. Sci Rep 6(1):26813. https://doi.org/10.1038/srep26813

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25(4):402–408. https://doi.org/10.1006/meth.2001.1262

McManaman JL, Neville MC (2003) Mammary physiology and milk secretion. Adv Drug Deliver Rev 55(5):629–641. https://doi.org/10.1016/S0169-409x(03)00033-4

Moyes KM, Drackley JK, Morin DE, Loor JJ (2010) Greater expression of TLR2, TLR4, and IL6 due to negative energy balance is associated with lower expression of HLA-DRA and HLA-A in bovine blood neutrophils after intramammary mastitis challenge with Streptococcus uberis. Funct Integ Genomic 10(1):53–61. https://doi.org/10.1007/s10142-009-0154-7

Nagaraj N, Wisniewski JR, Geiger T, Cox J, Kircher M, Kelso J, Paabo S, Mann M (2011) Deep proteome and transcriptome mapping of a human cancer cell line. Mol Syst Biol 7(1):548. https://doi.org/10.1038/msb.2011.81

Ouattara B, Bissonnette N, Duplessis M, Girard CL (2016) Supplements of vitamins B9 and B12 affect hepatic and mammary gland gene expression profiles in lactating dairy cows. BMC Genomics 17(1):640. https://doi.org/10.1186/s12864-016-2872-2

Ozsolak F, Milos PM (2011) RNA sequencing: advances, challenges and opportunities. Nature Rev Genet 12(2):87–98. https://doi.org/10.1038/nrg2934

Qiu XB, Shao YM, Miao S, Wang L (2006) The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. Cell Mol Life Sci 63(22):2560–2570. https://doi.org/10.1007/s00018-006-6192-6

Rawson P, Stockum C, Peng L, Manivannan B, Lehnert K, Ward HE, Berry SD, Davis SR, Snell RG, McLauchlan D, Jordan TW (2012) Metabolic proteomics of the liver and mammary gland during lactation. J Proteome 75(14):4429–4435. https://doi.org/10.1016/j.jprot.2012.04.019

Robinson SD, Silberstein GB, Roberts AB, Flanders KC, Daniel CW (1991) Regulated expression and growth inhibitory effects of transforming growth factor-beta isoforms in mouse mammary gland development. Development (Cambridge, England) 113:867–878

Rui L, Yuan M, Frantz D, Shoelson S, White MF (2002) SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem 277(44):42394–42398. https://doi.org/10.1074/jbc.C200444200

Seo M, Lee HJ, Kim K, Caetano-Anolles K, Jeong JY, Park S, Oh YK, Cho S, Kim H (2016) Characterizing milk production related genes in Holstein using RNA-seq. Asian-Austral J Anim 29(3):343–351. https://doi.org/10.5713/ajas.15.0525

Shi H, Zhu J, Luo J, Cao W, Shi H, Yao D, Li J, Sun Y, Xu H, Yu K, Loor JJ (2015) Genes regulating lipid and protein metabolism are highly expressed in mammary gland of lactating dairy goats. Funct Integr Genomic 15(3):309–321. https://doi.org/10.1007/s10142-014-0420-1

Sordillo LM (2005) Factors affecting mammary gland immunity and mastitis susceptibility. Livest Prod Sci 98:89–99. https://doi.org/10.1016/j.livprodsci.2005.10.017

Sordillo LM, Shafer-Weaver K, DeRosa D (1997) Immunobiology of the mammary gland. J Dairy Sci 80(8):1851–1865. https://doi.org/10.3168/jds.S0022-0302(97)76121-6

Stein T, Salomonis N, Nuyten DS, van de Vijver MJ, Gusterson BA (2009) A mouse mammary gland involution mRNA signature identifies biological pathways potentially associated with breast cancer metastasis. J Mammary Gland Biol Neoplasia 14(2):99–116. https://doi.org/10.1007/s10911-009-9120-1

Swanson KM, Stelwagen K, Dobson J, Henderson HV, Davis SR, Farr VC, Singh K (2009) Transcriptome profiling of Streptococcus uberis-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model. J Dairy Sci 92(1):117–129. https://doi.org/10.3168/jds.2008-1382

Taga H, Chilliard Y, Meunier B, Chambon C, Picard B, Zingaretti MC, Cinti S, Bonnet M (2012) Cellular and molecular large-scale features of fetal adipose tissue: is bovine perirenal adipose tissue brown? J Cell Physiol 227(4):1688–1700. https://doi.org/10.1002/jcp.22893

Team RC (2014) R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. Available online at: http://www.R-project.org

Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L (2014) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks (vol 7, pg 562, 2012). Nat Protoc 9(10):2513–2513. https://doi.org/10.1038/nprot1014-2513a

Walter W, Sanchez-Cabo F, Ricote M (2015) GOplot: an R package for visually combining expression data with functional analysis. Bioinformatics (Oxford, England) 31(17):2912–2914. https://doi.org/10.1093/bioinformatics/btv300

Wang WH, Morrison B, Galbaugh T, Jose CC,Kenney N, Cutler ML, (2008) Glucocorticoid induced expression of connective tissue growth factor contributes to lactogenic differentiation of mouse

mammary epithelial cells. J Cell Physiol 214(1):38–46.https://doi.org/10.1002/jcp.21159

Wang B, Mao S, Yang H, Wu Y, Wang J, Li S, Shen Z, Liu J (2014) Effects of alfalfa and cereal straw as a forage source on nutrient digestibility and lactation performance in lactating dairy cows. J Dairy Sci 97(12):7706–7715. https://doi.org/10.3168/jds.2014-7961

Whetstone HD, Hurley WL, Davis CL (1986) Identification and characterization of a fatty acid binding protein in bovine mammary gland. Comp Biochem Phys 85:687–692

Zhang F, Beharry ZM, Harris TE, Lilly MB, Smith CD, Mahajan S, Kraft AS (2009) PIM1 protein kinase regulates PRAS40 phosphorylation and mTOR activity in FDCP1 cells. Cancer Biol Ther 8(9):846–853. https://doi.org/10.4161/cbt.8.9.8210

Zheng Y, Chen KL, Zheng XM, Li HX, Wang GL (2014) Identification and bioinformatics analysis of microRNAs associated with stress and immune response in serum of heat-stressed and normal Holstein cows. Cell Stress Chaperones 19(6):973–981. https://doi.org/10.1007/s12192-014-0521-8