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Differential expression of genes in milk of dairy cattle during lactation

Jie Yang*, Jicai Jiang*, Xuan Liu*, Haifei Wang*, Gang Guo[†], Qin Zhang* and Li Jiang*

*Key Laboratory of Animal Genetics, Breeding and Reproduction, Ministry of Agriculture, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China. [†]Beijing Sanyuan Breeding Technology Co. Ltd., Beijing 100029, China.

Summary

The milk fat globule (MFG) is one of the most representative of mammary gland tissues and can be utilized to study gene expression of lactating cows during lactation. In this study, RNA-seq technology was employed to detect differential expression of genes in MFGs at day 10 and day 70 after calving between two groups of cows with extremely high (H group) and low (L group) 305-day milk yield, milk fat yield and milk protein yield. In total, 1232, 81, 429 and 178 significantly differentially expressed genes (false discovery rate $q < 0.05$) were detected between H10 and L10, H70 and L70, H10 and H70, and L10 and L70 respectively. Gene Ontology enrichment and pathway analysis revealed that these differentially expressed genes were enriched in biological processes involved in mammary gland development, protein and lipid metabolism process, signal transduction, cellular process, differentiation and immune function. Among these differentially expressed genes, 178 (H10 vs. L10), 4 (H70 vs. L70), 68 (H10 vs. H70) and 22 (L10 vs. L70) were found to be located within previously reported QTL regions for milk production traits. Based on these results, some promising candidate genes for milk production traits in dairy cattle were suggested.

Keywords differentially expressed genes, milk fat globules, milk production traits, RNA sequencing

Introduction

The mammary gland is the only organ that undergoes regular proliferation and involution cycles after maturity (Gao *et al.* 2013). Mammary epithelial cells (MECs) are unique in that they synthesize and secrete milk (Canovas *et al.* 2014). Knowledge of the molecular events in mammary epithelium cells during lactation would contribute to the development of new technologies in the management and breeding of dairy cattle. Numerous studies on gene expression in the bovine mammary gland have been carried out by performing mammary biopsies, which is invasive and costly and disturbs the normal lactation process. In addition, studies of metabolism in bovine mammary tissue are complicated due to the presence of multiple cell types within the gland, such as adipocytes, fibroblasts, blood vessels and immune cells (Dulbecco *et al.* 1982; Maningat *et al.* 2009; Van Keymeulen *et al.* 2011).

During lactation, milk fat is synthesized in MECs (Lemay *et al.* 2007). MECs secrete fat into milk via an apocrine mechanism along with a crescent of the MEC cytoplasm enveloped in plasma membrane, resulting in the secretion of milk fat globules (MFGs) (Patton & Huston 1988; McManaman *et al.* 2004). Intracellular components of the MECs become trapped within the MFGs (Huston & Patton 1990). Thus, the RNA from MFGs is expected to represent RNA from MECs at the time they are producing milk, as demonstrated by a number of previous studies (Maningat *et al.* 2007, 2009; Brenaut *et al.* 2012). Canovas *et al.* (2014) also suggested that the MFG transcriptome is the most representative of the transcriptome of mammary gland tissue and laser microdissected MECs. Therefore, MFGs in milk can be utilized as effective and easily obtainable sources to study gene expression during lactation.

In the current study, we examined differential expression of genes in MFGs of lactating cows with remarkably different milk production performance using RNA sequencing (RNA-seq) technology.

Materials and methods

Animals and milk samples

Based on their milk production records in their previous lactation, six Chinese Holstein cows, of which three were in

Address for correspondence

L. Jiang, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China.
E-mail: lijiang@cau.edu.cn

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their second and three in their third lactation, were selected from the Beijing Sanyuanlvhe Dairy Farm. The six cows were divided into two groups, the H group and L group, each with three cows according to their performance in their previous lactation. The cows in the H group had higher 305-day milk yield (MY > 11 000 kg), fat yield (FY > 410 kg) and protein yield (PY > 340 kg), whereas cows in the L group had lower MY, FY and PY (<8600 kg, 360 kg and 300 kg respectively). Detailed information about the six cows is shown in Table 1. The age differences among the three cows in the second lactation and among the three cows in the third lactation were less than 45 days and less than 20 days respectively. Among the six cows, there were two pairs of half sibs, each pair consisting of one H cow and one L cow. The other two cows, one H and one L cow, were non-sibs.

Milk samples were collected from these cows at two lactation stages: the early stage (days in milk = 10) and peak stage (days in milk = 70). Hereafter, cows of the H group at early stage and peak stage are referred to as H10 and H70 respectively, and cows of the L group at early stage and peak stage are referred to as L10 and L70 respectively. Milk samples were collected into sterile, RNase-free bottles before the morning milking and then kept on ice prior to the MFG collection.

Collection of MFGs

Approximately 45 ml of milk was transferred into sterile, RNase-free 50-ml tubes and then centrifuged (Eppendorf, Germany) at 3000 ***g*** for 10 min at 4 °C. The supernatant fat layer was transferred into a new, RNase-free, pre-weighed 15-ml tube using an RNase-free spoon. Twelve milliliters (12 ml) of TRIzol reagent (Invitrogen Life Technology) was added to dissolve the fat layer prior to storing at −80 °C.

RNA isolation and sequencing

Total RNA was extracted from the 12 milk samples. RNA isolation was performed using the RNeasy method (Qiagen), following the manufacturer's protocols. RNA concentration was measured via a ND-2000 Spectrophotometer (Thermo Scientific). The quality of the total RNA was evaluated using the RNA integrity number (RIN) value in an Agilent Technologies 2100 Bioanalyzer. The RIN value of each sample was around 7.0. Messenger RNA was isolated and purified using a Dynabeads mRNA DIRECT Kit (Invitrogen Life Technology). Then, mRNA was fragmented and first- and second-strand cDNA were synthesized. After end repair and adapter ligation, a 400- to 500-bp fragment size was selected by gel excision and each sample was individually sequenced on the Illumina Hiseq 2500 platform.

Table 1 Information about animals used in this study

Animal[1]	Parity	305-day milk yield (kg)	Milk fat yield (kg)	Milk protein yield (kg)
H-1	3	12 514	472.28	394.19
H-2	3	12 966	466.78	414.91
H-3	2	11 222	419.70	345.64
L-1	3	8565	358.87	298.06
L-2	2	6184	267.77	207.16
L-3	2	7837	355.02	282.13

[1]H-1 to H-3, animals in the H (high yield) group; L-1 to L-3, animals in the L (low yield) group.

Detection of differentially expressed genes

Based on the bovine reference genome (UMD3.1) (ftp://ftp.ensembl.org/pub/release-72/fasta/bos_taurus/dna/) and the corresponding gene model annotation files (ftp://ftp.ensembl.org/pub/release-72/gtf/bos_taurus/), clean reads for each sample were mapped to the reference genome by TOPHAT (v2.0.10) and assembled by CUFFLINKS (v2.1.1) (http://cole-trapnell-lab.github.io/cufflinks/). Then, CUFFDIFF, which is included in the CUFFLINKS package, was used to detect differentially expressed genes and transcripts between the H and L groups at the same stage of lactation (H10 vs. L10, H70 vs. L70) as well as between early and peak stage of lactation in the same group (H10 vs. H70, L10 vs. L70). A significantly differential expression was declared if the q-value (false discovery rate corrected P-values) was <0.05. In addition, we performed Gene Ontology (GO) enrichment analysis and pathway analysis of the differentially expressed genes using DAVID software v6.7 (http://david.abcc.ncifcrf.gov/). The GO terms and pathways were considered significant if their Benjamini corrected P-values were <0.05.

Results

Mapping and annotation

After removal of sequencing adaptors and low-complexity reads, we acquired 60 204 905–93 829 745 paired-end reads per sample. TOPHAT software (http://ccb.jhu.edu/software/tophat/index.shtml) was employed to map the reads against the bovine reference genome. The observed percentages of mapped reads per sample were around 64.17–75.61%, and the percentages of unique aligned reads per sample were around 65.92–89.97% (Table S1). PICARD (http://broadinstitute.github.io/picard/) was used to calculate the proportion of reads mapping to coding regions, introns, 5′-/3′-UTR regions and intergenic regions. As expected, the highest percentage of reads mapped to coding regions (70.83–80.74%), whereas 16.34–21.72% mapped to 5′-/3′-UTR regions, 2.81–10.50% mapped to intergenic regions and only 0.11–0.62% mapped to introns (Table S2). All the raw sequence data were deposited in SRA (GenBank Accession number SRP064718).

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Differentially expressed genes between H and L groups

In total, 1232 significantly differentially expressed genes were detected between H10 and L10, whereas only 81 genes were identified to be significantly differentially expressed between H70 and L70 (Table 2). Most of the differentially expressed genes (1076 of 1232 and 78 of 81 respectively) showed higher expression in the cows of the L group (Fig. 1). Furthermore, of the 81 genes differentially expressed between H70 and L70, 71 were also differentially expressed between H10 and L10. Details of the differentially expressed genes are shown in Tables S3 and S4.

Differentially expressed genes between early and peak stages of lactation

In the H group, 429 genes were identified to be significantly differentially expressed between the early and peak stages of lactation, 220 (51.3%) of which were downregulated at the peak stage of lactation (Table 2, Fig. 2a). In the L group, 178 genes were significantly differentially expressed between the early and peak stages of lactation, and 162 (91.01%) of them showed lower expression at the peak stage of lactation (Table 2, Fig. 2b). In addition, 90 significantly differentially expressed genes overlapped between the two comparisons. Details of the differentially expressed genes are shown in Tables S5 and S6.

Table 2 Summary of the differentially expressed (DE) genes in the four comparisons

Comparison[1]	No. DE genes	Upregulated genes in L group or at early stage
H10 vs. L10	1232	1076 (87.3%)
H70 vs. L70	81	78 (96.3%)
H10 vs. H70	429	220 (51.3%)
L10 vs. L70	178	162 (91.0%)

[1]H and L refer to the high-yield and low-yield group respectively; 10 and 70 refer to the early (days in milk = 10) and peak (days in milk = 70) stage of lactation respectively.

Distribution of differentially expressed genes

The distribution of the differentially expressed genes in the four comparisons (H10 vs. L10, H70 vs. L70, H10 vs. H70 and L10 vs. L70) is shown in Fig. 3. The largest number of differentially expressed genes was observed between H10 and L10, whereas the smallest number was observed between H70 and L70. Fifteen of the differentially expressed genes (Fig. 3) were detected in all four comparisons.

Gene Ontology enrichment and pathway analysis

The GO enrichment analysis of the differentially expressed genes revealed 55, 4 and 29 significant GO terms (Benjamini corrected $P < 0.05$) for the comparisons of H10 vs. L10, H70 vs. L70 and H10 vs. H70 respectively (Tables S7–S9). These GO terms are involved in mammary gland development, protein and lipid metabolism process, signal transduction, cellular process, differentiation and immune function. At the same time, the pathway analysis revealed two significant pathways, bta04060 (cytokine–cytokine receptor interaction) for H70 vs. L70 (Table S8) and bta04621 (NOD-like receptor signaling pathway) for H10 vs. H70 (Table S9).

Integrated analysis of RNA-seq and animal QTLdb

We integrated the differentially expressed genes and the QTL (quantitative trait loci) for milk production traits collected in the QTLdb database (http://www.animalgenome.org/cgi-bin/QTLdb/BT/index), which were detected by either QTL mapping studies or genome-wide association studies (GWAS), by comparing their chromosome positions in order to gain further insights into the association of the differentially expressed genes with milk production traits. For QTL detected by QTL mapping studies, only those with a confidence interval less than 1 Mb were considered as a QTL region. For QTL revealed by GWAS, the 200 kb upstream/downstream of the significant SNP were defined as a QTL region. Among the differentially expressed genes from the four comparisons, 178 (H10 vs. L10), 4

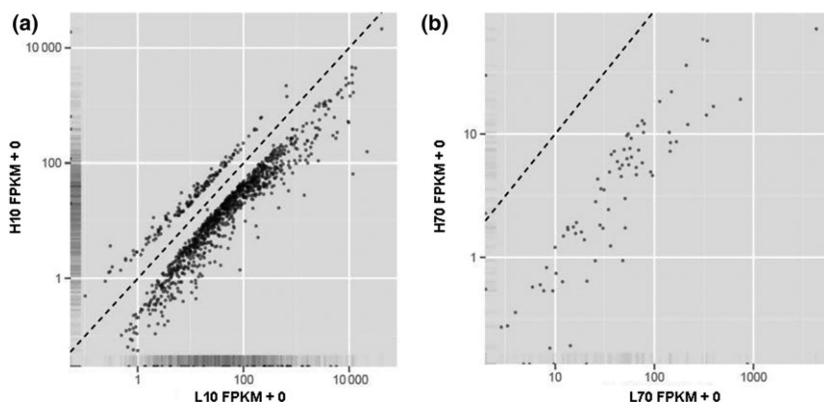

Figure 1 Differential expression of genes between the high (H) and low (L) groups. FPKM values under (above) the dotted line represent higher expression in the L(H) group. (a) H10 vs. L10; (b): H70 vs. L70.

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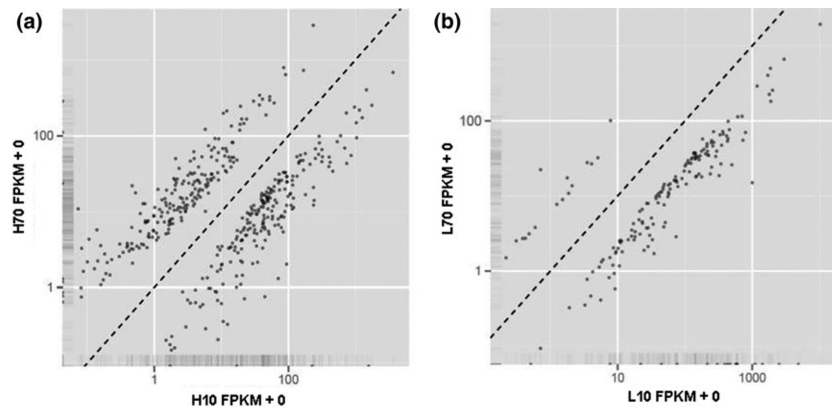

Figure 2 Differential expression of genes between the early (days in milk = 10) and peak (days in milk = 70) stage of lactation. FPKM values under (above) the dotted line represent higher expression at the early (peak) stage of lactation. (a) H10 vs. H70; (b) L10 vs. L70.

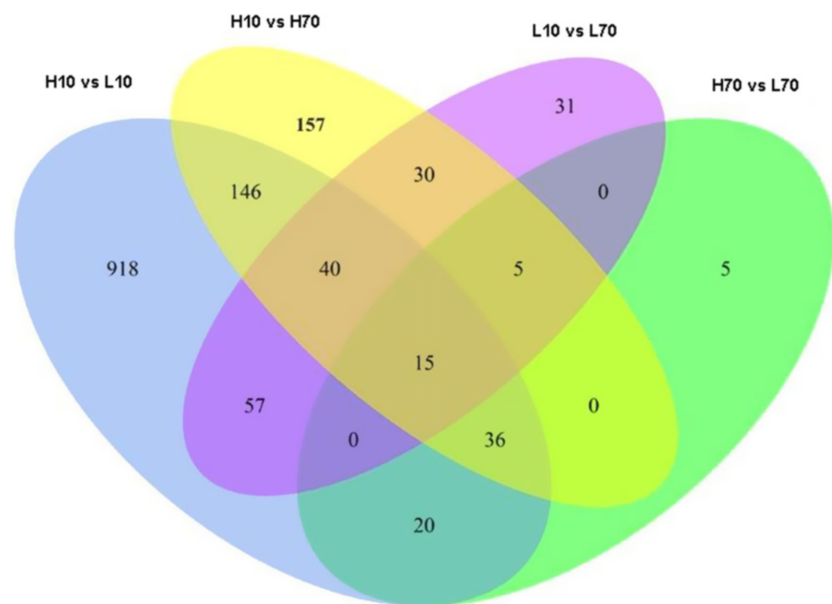

Figure 3 Overlapping of differentially expressed genes from the four comparisons (H10 vs. L10, H70 vs. L70, H10 vs. H70 and L10 vs. L70).

(H70 vs. L70), 68 (H10 vs. H70) and 22 (L10 vs. L70) genes were found to be located within QTL regions (Table S10).

Discussion

To identify candidate genes for milk production traits, it is important to reveal the transcriptome profiles of MECs during lactation. Most of previous studies on gene expression during lactation in dairy cattle were based on mammary gland tissue taken by mammary biopsy (Hou *et al.* 2010; Gao *et al.* 2013). However, the mammary gland tissue contains not only MECs but also myoepithelial cells and mesenchymal cells, including adipocytes, fibroblasts, blood vessels and immune cells (Dulbecco *et al.* 1982; Van Keymeulen *et al.* 2011). Thus, RNA isolated from mammary gland tissue dose not accurately describe the gene expression in mammary epithelium cells. In contrast, MFGs come solely from MECs (Maningat *et al.* 2009); therefore, RNA isolated from MFGs is better representative of RNA than that from MECs. Furthermore, for studying gene expression during lactation, using MFGs is much easier than using MECs because MFGs can be easily collected at any time during lactation, whereas collecting mammary gland tissue by mammary biopsy is invasive and costly and disturbs the normal lactation process.

A large number of differentially expressed genes were identified by RNA-seq. Most of the differentially expressed genes showed lower expression in the cows with higher MY, FY and PY, that is, cows in the H group or at the peak stage (Table 2). In particular, 91.01% of the differentially expressed genes between L10 and L70 showed lower expression at the peak stage of lactation. Similar results were obtained by Cui *et al.* (2014), who studied the differential expression of genes in mammary gland tissue of lactating cows with extremely high and low fat and protein percentage. They found that most of the differentially expressed genes (28 of 31) were downregulated in the cows with higher fat and protein percentage. This is consistent with our results given that fat and protein

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percentage are positively correlated with FY and PY respectively. It has been commonly observed in bovine (Finucane *et al.* 2008; Bionaz *et al.* 2012; Gao *et al.* 2013) as well as in other mammals (Rosen *et al.* 1975; Shuster *et al.* 1976; Robinson *et al.* 1995; Rijnkels *et al.* 1997) that many more differentially expressed genes are downregulated than are upregulated in mammary gland in early lactation compared to in late pregnancy. It was also noted that these downregulated genes are largely involved in cell division, cell cycle and their related processes. Because the mammary gland cell proliferation occurs mainly in late pregnancy period and almost ceases during the lactation period (Sorensen *et al.* 2006), it is reasonable to expect that many genes involved in cell proliferation and other related biological processes may be already expressed at a high plateau level at the late pregnant stage and decrease their expression or turn off in the lactation period. This explanation may also apply to our observation, that is, that most of the differentially expressed genes showed lower expression in the cows with higher milk production performance. Most of these downregulated genes were also enriched in cell process, activation, communication and proliferation. Their decreased expression may be coupled with increased expression of genes involved in metabolism and transporter activities, leading to higher milk production performance.

The GO enrichment analysis revealed that the differentially expressed genes are enriched in a large number of GO terms; some of them are involved in mammary gland development, protein and lipid metabolism process, signal transduction, cell cycle/death, differentiation and immune function. Here, we focus on two of these GO terms, GO:0006631 (fatty acid metabolic process) and GO:0030879 (mammary gland development), which are more likely related to milk production traits due to their biological function. Differentially expressed genes included in these two terms are presented in Table 3. Among these genes, *STAT5A*, *PRLR*, *NCF1* and *PTGES* are located within reported QTL regions (Table S10) and are considered promising candidate genes for milk production traits. STAT5A is a mediator of prolactin signaling in MECs and is known to play a role in lactogenesis (Wheeler *et al.* 2001). Khatib *et al.* (2008) reported the association of the bovine *STAT5A* gene with fertilization success, embryonic survival and milk composition. Liu *et al.* (1997) found that STAT5A–/– mice were normal in appearance, size, weight and fertility, whereas their mammary development was impaired and females failed to lactate. PRLR is a specific membrane receptor for the prolactin hormone (PRL), which plays an important regulatory function in mammary gland development, expression of milk protein genes and lactation (Kelly *et al.* 2002). Kelly *et al.* (2001, 2002) found that homozygous *PRLR* knockout mice (*PRLR–/–*) were completely infertile due to a complete failure of blastocysts to implant and lack of normal mammary development. In progesterone-treated mice, pregnancy was rescued but the mammary gland was severely underdeveloped. *NCF1* is involved in inflammatory response. Efimova *et al.* (2011) found that NCF1 was essential for direct regulatory T-cell-mediated suppression of CD4+ effector T cells. PTGES is a member of the membrane-associated proteins in the eicosanoid and glutathione metabolism family and involved in arachidonic acid metabolism (Mancini *et al.* 2001).

Table 3 Differentially expressed genes in GO:0030879 and GO:0006631

GO term	Differentially expressed genes
GO:0030879—mammary gland development	*IRF6, AGPAT6, STAT5A, XDH, B4GALT1, BCL2L11, PRLR*
GO:0006631—fatty acid metabolic process	*PRKAA1, NCF1, AGPAT6, STAT5A, CRYL1, GPAM, ALOX12, PGHS-2, PRKAR2B, CPT1B, SLC27A1, FADS6, PPARD, ACACA, PTGES, EHHADH, ALOX5*

GO, Gene Ontology.

Although the other genes in these two terms are not located within the reported QTL regions, they may also be good candidate genes for milk production traits. For example, *AGPAT6* encodes a protein that is involved in both fatty acid metabolic process and mammary gland development and belongs to the 1-acylglycerol-3-phosphate O-acyltransferase (*AGPAT*) family. Beigneux *et al.* (2006) reported that the alveoli and ducts of lactating mammary glands of *AGPAT6–/–* mice were underdeveloped. Also, *AGPAT6–/–* mice had a significant decrease in the size and number of lipid droplets within the MECs and ducts. In addition, the milk from *AGPAT6–/–* mice was markedly reduced in diacylglycerol and triacylglycerol. These observations indicated that AGPAT6 is crucial for milk production. In addition, the genes *EHHADH*, *FADS6*, *ALOX5*, *ALOX12* and *ACACA* are all involved in fatty acid synthesis and metabolism (Horrobin 1993; Mehrabian *et al.* 2008; Houten *et al.* 2012; Matsumoto *et al.* 2012; Wang *et al.* 2012; Habermann *et al.* 2013).

In conclusion, the present study provided important information on differentially expressed genes between cows with high and low milk production performance at early and peak stage of lactation using RNA-seq technology and RNA from MFGs. Integrated analysis of differential gene expression, QTL/GWAS data and GO enrichment analysis suggested that some of the differentially expressed genes could be considered promising candidate genes for milk production traits in dairy cattle.

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Competing interests

The authors declare that they have no competing interests.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Numbers of reads obtained and percentages of mapped reads per sample

Table S2 Proportions of reads mapping to coding, intron, 5′/3′UTR or intergenic regions

Table S3 Details of differentially expressed genes between H10 and L10

Table S4 Details of differentially expressed genes between H70 and L70

Table S5 Details of differentially expressed genes between H10 and H70

Table S6 Details of differentially expressed genes between L10 and L70

Table S7 Summary of the Gene Ontology enrichment and pathway analysis of differentially expressed genes between H10 and L10

Table S8 Summary of the Gene Ontology enrichment and pathway analysis of differentially expressed genes between H70 and L70

Table S9 Summary of the Gene Ontology enrichment and pathway analysis of differentially expressed genes between H10 and H70

Table S10 Details of differentially expressed genes located within reported QTL regions for milk production traits