Biochemical and Biophysical Research Communications xxx (2017) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Quantitative proteome analysis of bovine mammary gland reveals protein dynamic changes involved in peak and late lactation stages

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ARTICLE INFO

Article history:
Received 28 September 2017
Accepted 7 October 2017
Available online xxx

Keywords:
Proteome
Cow
Mammary gland
TMT
Different lactation stages

ABSTRACT

Mammary gland is an important organ for milk synthesis and secretion. It undergoes dramatic physiological changes to adapt the shift from peak to late lactation stage. Protein plays a final very vital role in many life functions, and the protein changes during different lactation stages potentially reflect the biology of lactation and the functions of mammary gland in cows. In current study, we adopted tandem mass tags label-based quantitative analysis technique and to investigate proteome changes occurring in bovine mammary gland from peak to late lactation stages. A total of 3753 proteins from mammary tissues taken at two lactation points from four individual cows by biopsy were quantified, out of which 179 proteins were expressed differentially between two stages. We observed five new DEPs (AACS, DHCR7, GSTM3, SFRP1 and SFRP4) and nine functional well-studies known proteins (PLIN2, LPIN1, PLIN3, GSN, CD74, MMP2, SOD1, SOD3 and GPX3) related to milk performance and mammary morphology. Bioinformatics analyses of the DEPs showed a majority of the up-regulated proteins during late lactation stage were related to apoptosis and immune process, while the downregulated proteins were mainly involved in localization, lipid metabolic and transport process. This suggests that the mammary gland can adapt to different molecular functions according to the biological need of the animal. From the integrated analysis of the differentially expressed proteins with known quantitative trait loci and genome-wide association study data, we identified 95 proteins may potentially affect milking performance. We expect findings in this study could be a valuable resource for future studies investigating the bovine proteome and functional studies.

1. Introduction

Lactation is a multifactorial complex process as well as a key stage of mammalian reproductive physiology. The processes of lactation include the proliferation and involution cycles of mammary gland, as well as the synthesis and secretion of milk [1,2]. It was widely considered that peak yield and length of peak lactation stage can affect the total milk production directly, *e.g.*, if peak yield increases 1 kg/day, the total milk yields will improve 250–400 kg [3]. Therefore manipulation of lactation performance and understanding the biological pathways and mechanisms that govern mammary gland development and lactation is commercially important [4].

Mammary glands of dairy cattle are specialized in producing milk for the newborn offspring and for human consumption [5]. It undergoes regular proliferation and involution cycles after maturity [6]. The dynamic nature of mammary makes it an ideal model for the research of molecular mechanisms involved in cell proliferation and differentiation as well as organ development [7]. Besides, the development of mammary gland is coordinated with important functional events, which are mediated by hormone and complex shifts in proteins [8]. Proteins are the direct bio-functional molecules in the living organisms [9]. The knowledge of the protein changes during different lactation stages can reflect the physiological and metabolic changes of mammary structure [10]. In recent years, several research groups have reported the dynamic changes at protein level during lactation in rats [11] and cattle [12,13]. Most

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https://doi.org/10.1016/j.bbrc.2017.10.038
0006-291X/

of these protein studies are based on 2D gel-based assay. Although 2D gel-based assay is a well-developed technology to perform proteomics analyses, Tandem Mass Tags (TMT) is a more precise and sensitive technique to study and quantify multiplexed peptide/protein with mass spectrometry in large-scale [14]. Even though some studies have reported the changes at proteome level of bovine, most of the researches were focused on non-mammary tissues, such as bovine milk or mammary epithelia cells [12,15]. However, alterations of mammary proteins during different lactation stages have not been studied extensively, especially by isobaric mass tagging technique (*e.g.*, TMT and iTRAQ).

The goal of this study was to explore the proteome changes of Chinese Holsteins' mammary glands between peak and late lactation stage. We performed biopsy experiment to obtain mammary tissues in peak and late lactation stage, which avoided different genetic background among four cows. TMT-based proteomic method provides high coverage of mammary proteome as well as valid screening of differentially expressed proteins between two stages of lactation. Further bioinformatics and integrative analyses were carried out to reveal DEPs related to lactation performance. The investigation of alterations of mammary protein during different lactation stage in cow will provide resources for further function studies in bovine mammary for exploring the mechanism of lactation and identify potential biomarkers affecting milk quality and milk production traits.

2. Materials and methods

2.1. Ethics statements and animals

All the experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of China Agricultural University.

Four multiparous and healthy, mastitis-free Chinese Holstein cows at the Zhonghe Dairy Farm (Taiyuan, Shanxi Province, China) were selected for the study. Two cows were in their 2nd and the other two were in 3rd parity when mammary gland tissue was collected. The 305-day milk yield, and milk fat and protein percentages in the milk of the four cows were 6832–8028 kg, 3.36–3.41%, and 3.64–4.18%, respectively. The animals were kept in free stall housing, fed total mixed ration (TMR) and had access to water ad libitum. Cows were milked three times daily in the milking parlor. All animals were fed the same diet and kept in the same location during the experimental period.

2.2. Mammary gland biopsies

Mammary gland tissue biopsies were taken from the four Chinese Holstein cows at two lactation stages: peak stage (P, days 90 postpartum) and late stage (L, days 270 postpartum) of lactation. We performed the biopsy experiment according to previous studies [16,17]. When the experiment finished, all four cows received antibiotic prophylaxis and anti-inflammatory therapy immediately. Biopsy wounds healed and milk yield returned to pre-biopsy values within 3 days. It has been demonstrated the dairy cow could recovery rapidly post-biopsy, which did not affect the cow's healthy [18].

2.3. Protein extraction and TMT

After protein extraction, Tandem mass tags TMT^{10} (Pierce, USA) with different reporter ions (126–131 Da) were applied as isobaric tags for relative quantification. TMT labeling was performed according to the manufacturer's instructions. Detailed methods were provided in Supplementary Material.

2.4. LC-MS/MS analysis and data processing

The liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis was carried out by Q Exactive mass spectrometer (Thermo Scientific, CA). All the tandem mass spectra were produced by higher-energy collision dissociation (HCD) method. Detailed methods and analysis parameters were provided in Supplementary Material. LC-MS/MS raw data were processed using Mascot search engine 2.5.1 (Matrix Science) [19,20] and X! Tandem CYCLONE against the *Bos taurus* sequences in UniprotKB, version 2016_07, for a total of 19,382 entries. Mascot and X!Tandem were searched with a fragment ion mass tolerance of 0.050 Da and a parent ion tolerance of 10.0 PPM. Scaffold 4.6.2 (Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. A 1% FDR was required at both the protein level and the peptide level. Minimum required peptide length was 6 amino acids for both identification and quantification. A minimum of 2 unique peptides for each protein were required for reliable identification.

Differential expression was assessed using the Limma package in R/Bioconductor [21]. Proteins with a P value of less than 0.05 and an absolute fold change more than 1.2 were considered expressed differentially between peak and late lactation stages.

2.5. Bioinformatics analysis

Considering the limitation of the GO annotation of genes in the bovine genome, we had converted the cow refGene/Ensembl gene IDs to orthologous human EntrezGene IDs by BioMart (http://www.ensembl.org/biomart/). We used PANTHER classification system (http://www.pantherdb.org/) and KOBAS 3.0 to identify significantly enriched GO terms and pathways (http://kobas.cbi.pku.edu.cn/anno_iden.php). A P value of < 0.05 determined by Fisher's exact test was set as the criteria for significance. In order to explore the relationship between the DEPs with milk composition and milk production traits, we integrated them with previously reported QTL mapping and GWAS data (http://www.animalgenome.org/cgi-bin/QTLdb).

2.6. Western-blotting analysis for validation

Sample homogenate was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. Membranes were subsequently incubated with four antibodies: SFRP4 (1:1000, ab154167, Abcam Biotech, Cambridge, U.K.), AACS (1:1000, ab128301, Abcam Biotech), Perilipin 2 (1:1000, ab52355, Abcam Biotech) and Actin (1:1000, ab8226, Abcam Biotech). Mouse monoclonal anti-beta actin antibody was used as loading control to normalize the amount of protein loaded in each sample. Protein bands were visualized and analyzed by densitometry using the NIH ImageJ software [22]. We analyzed the normalized western blot data by Student's t-test. Significant differences were considered when $P < 0.05$.

3. Results

To investigate the molecular basis of difference from peak lactation to late lactation, we used TMT labeling and mass spectrometry-based quantitative proteome approach to explore the molecular dynamics of mammary gland of Chinese Holstein cows between two typical different lactation stages, peak and late lactation stage (Fig. 1).

Please cite this article in press as: X. Zheng, et al., Quantitative proteome analysis of bovine mammary gland reveals protein dynamic changes involved in peak and late lactation stages, Biochemical and Biophysical Research Communications (2017), https://doi.org/10.1016/j.bbrc.2017.10.038

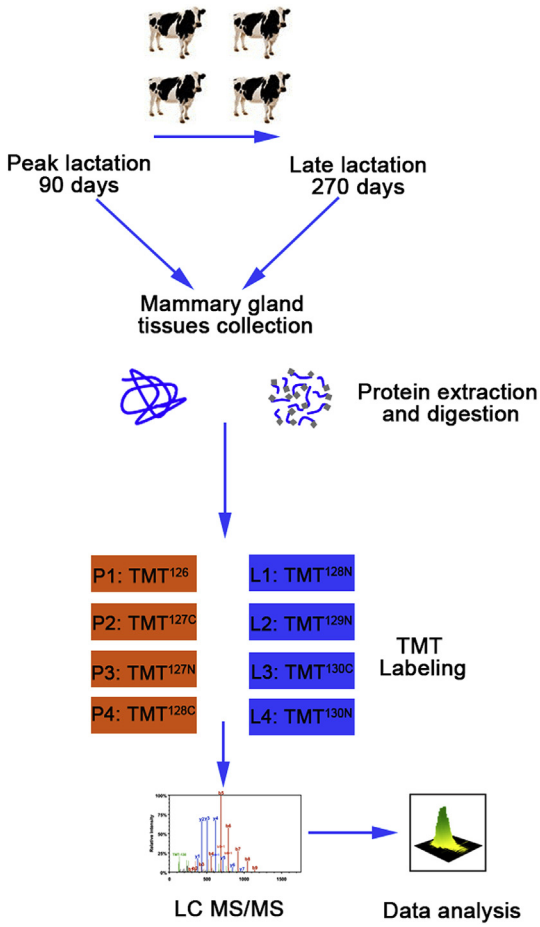

Fig. 1. Experimental study design.
Mammary gland tissues were collected at two lactation stages from four multiparous and healthy Chinese Holstein cows by biopsy. Proteome analysis was conducted using tandem mass tags (TMT) label-based quantitative technique.

3.1. Quantitative proteomics in mammary gland tissue of Chinese Holstein cow

In total, we identified 3753 proteins (Supplementary Table S1) by Mascot software, including 51,543 non-redundant peptides and with a highly conservative threshold (Confidence ≥ 95%, at less 2 unique peptides matched and FDR < 1%). Specifically, we confirmed protein evidence for 3753 out of 32,167 protein isoforms (11.67%) deposited in UnirpotKb. The peptide and protein score distributions are showed in Figure S1. The total proteins covered a broad range of protein classes, such as enzymes, cytoskeletal protein, extracellular protein and transcription factors (Fig. 2A).

3.2. Protein expression differences of mammary tissue between peak and late lactation stage

We observed 179 out of all identified proteins were differentially expressed between peak and late lactation stage of mammary gland (FC > 1.2 or FC < 0.83 as well as $P < 0.05$) (Supplementary Table S2). Compared with peak lactation stage, 69 proteins were significantly down-regulated (<0.83-fold) while 110 proteins were up-regulated (>1.2-fold) in late lactation stage (Fig. 2B). The top 10 differentially expressed proteins with the maximal fold change at each stage were showed in Table 1. From peak lactation to late lactation, milk yield declined accompanied by corresponding physiological changes in bovine mammary gland as well as changes in milk content [23]. We observed proteins related to milk lipid synthesis and mammary morphology, such as, phosphatidate phosphatase (LPIN1), perilipin-2 (PLIN2), perilipin-3 (PLIN3), 7-dehydrocholesterol reductase (DHCR7), acetoacetyl-CoA synthetase (AACS), gelsolin (GSN), frizzled-related protein 1 (SFRP1), frizzled-related protein 4 (SFRP4), GPX3, SOD1 and SOD3 were highly relevant with different lactation stages, which agrees with previous research results [12,24].

3.3. Bioinformatics analysis of the differentially expressed proteins

We divided the DEPs into three categories, i.e., Molecular

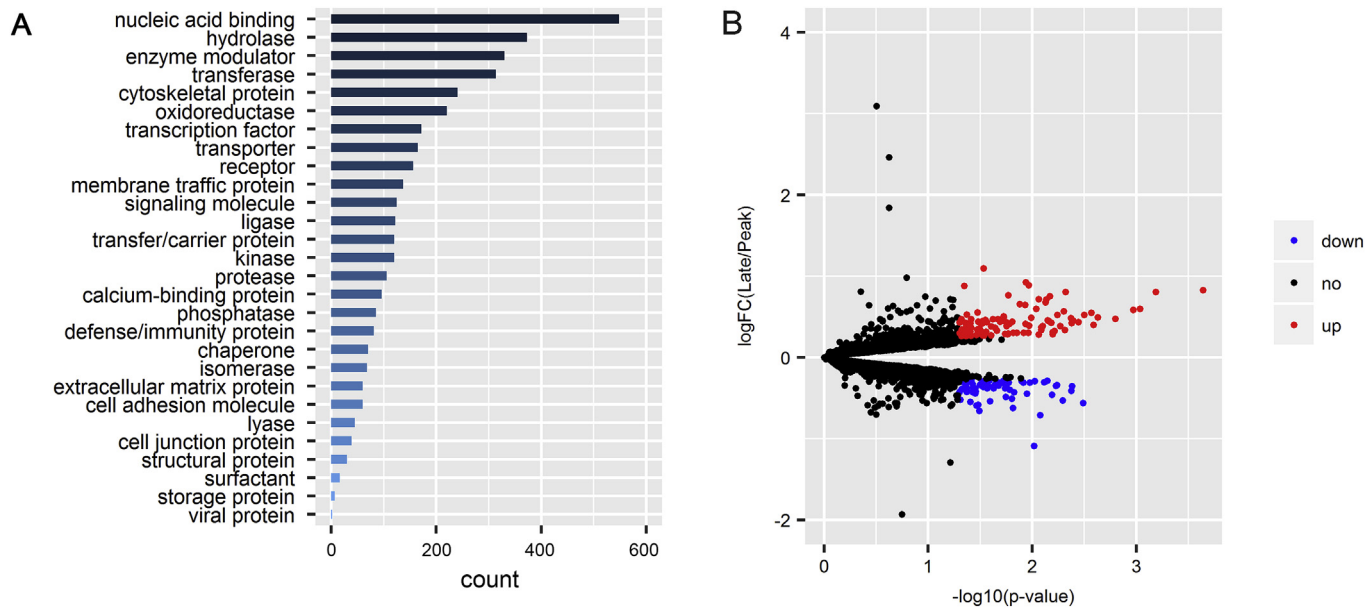

Fig. 2. Proteomic comparison of mammary gland between peak and late lactation stage.
(A) Classification of identified proteins. Bars show the number of proteins within each functional class.
(B) Differential proteins expression. Proteins expressed significantly higher ($P < 0.05$ and an absolute fold change > 1.2) in late and peak lactation are shown in red and blue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Table 1
Top 10 differentially expressed proteins between two lactation stages.

Category	ID	Gene symbol[a]	Fold change	*P* value
Down-regulated in peak lactation stage	G3X6Q8	PTX3	0.47	0.009
	F1MHQ1	GPA33	0.61	0.008
	F1MGR1	SLC28A3	0.65	0.015
	F1MUX6	GSTM3	0.66	0.034
	F1MUJ6	SLC1A2	0.67	0.033
	E1BP80	ZNF185	0.68	0.003
	Q2KIT8	TSTA3	0.69	0.025
	F1MHI1	PLIN2	0.69	0.005
	F6RF48	SFT2D2	0.70	0.049
	F1N3J3	AGR2	0.70	0.016
Up-regulated in late lactation stage	F1MFY5	C2CD3	1.57	0.013
	E1BI02	FMOD	1.60	0.007
	E1BA17	COL14A1	1.69	0.007
	F1MQX1	SRPX	1.70	0.017
	F1MX63	PRELP	1.75	0.0006
	Q2KHZ6	CD48	1.75	0.004
	A4IFB3	PLIN1	1.84	0.045
	A4IFE5	LRRC8D	1.85	0.011
	F1N583	OLFML1	1.90	0.011
	Q17QP5	SFRP4	2.14	0.029

[a] Gene symbol according to NCBI Entrez gene database http://www.ncbi.nlm.nih.gov/gene/.

Function (MF), Cellular Component (CC) and Biological Process (BP) (Figure S2). The DEPs in MF were mainly involved in catalytic activity (40%). The top three GO terms for BP were cellular process (26.6%), metabolic process (20.8%) and developmental process (10.20%). GO based on CC revealed that 34% and 18.6% of DEPs belonged to cell part and organelle, respectively.

We observed 919 GO terms and 23 KEGG pathways were significantly enriched ($P < 0.05$) (Supplementary Table S3), including metabolic process related to protein and lipid metabolism, transport, terms associated with the cell cycle and cell apoptosis and immune system process (Supplementary Table S4). Clearly, the overrepresented GO terms were different during peak and late lactation stage (Fig. 3).

The results also showed that 95 out of 179 DEPs (53.07%) were located within the reported QTL regions underlying milking performance including milk fat percentage, milk fat yield, milk protein percentage, milk protein yield and milk yield (Supplementary Table S5 and Supplementary Table S6). Specially, five out of the top 10 differentially expressed proteins with the maximal fold changes at each stage, glutathione S-transferase mu 3 (GSTM3), tissue-specific transplantation antigen P35B (TSTA3), SFRP4, olfactomedin-like protein (OLFML1) and CD48 molecule (CD48) were included.

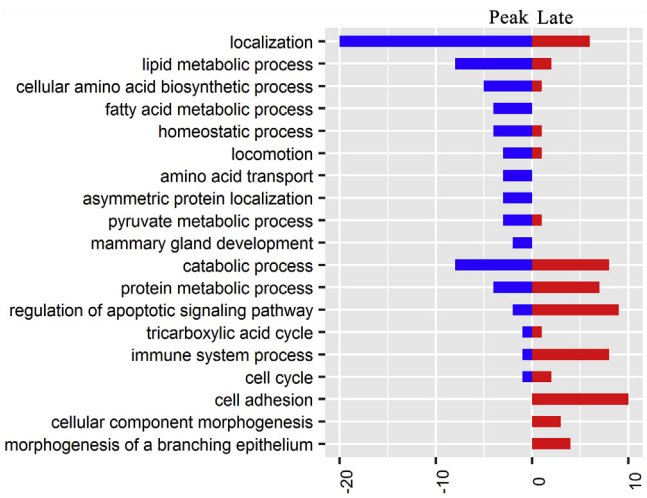

Fig. 3. Overrepresented biological processes of differentially expressed proteins. Red: late lactation; blue: peak lactation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Validation of important DEPs by western blotting

To validate the reliability of the proteomic results, we further performed Western blotting to detect the changes of important DEPs in mammary gland tissues (Fig. 4). DEPs involved in lactation and milk production traits were selected for validation. We detected the protein expression pattern of three important DEPs, i.e., SFRP4, AACS and PLIN2. The results showed that the change patterns of the three DEPs were in accordance with the results of TMT method, confirming the high quality and reliability of our TMT data.

4. Discussion

In current study, we investigated dairy cows' mammary gland proteome and identified 179 DEPs between two important lactation stages using biopsy longitudinal strategy. We found the up-regulated differentially expressed proteins during peak lactation stage were most involved in increasing milk yield and milk compositions while the up-regulated proteins during late lactation stage mainly associated with changes of mammary environmental, cell apoptosis and protection for mammary gland. The alterations of mammary proteins between peak and late lactations provided promising avenues for future research on the bovine functional studies.

Previous studies largely investigated dynamic transcriptome and proteome profiling in mammary gland among different lactation stage [13,17], where different experimental individuals were involved in distinct lactation stages. This maybe incurred false positive findings due to the confounding of individual genetic background and lactation stage. The use of longitudinal biopsies allowed us to observe protein alterations in the gland tissue of the identical animal more directly between peak and late lactation stages, avoiding the influence of different genetic backgrounds on the DEP detection. It is reasonably speculated that the fold changes of DEPs under such design in current were smaller than those using traditional comparison of groups with different lactation stages.

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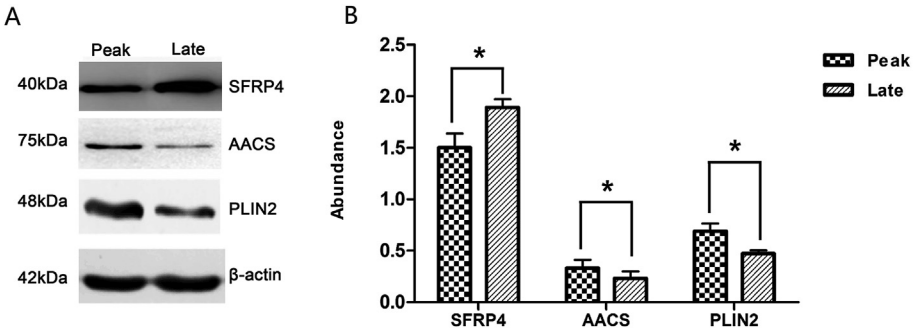

Fig. 4. Relative expression of SFRP4, AACS and PLIN2 in bovine mammary tissue between the two lactation stage.
(A) Comparison of protein abundance between the two stage by Western blotting of SFRP4, AACS and PLIN2.
(B) Bar graphs shows the densitometric analysis of the abundance of three differentially expressed proteins normalized to beta-actin in mammary gland tissue during peak and late lactation. Error bars represent SD. The abundance of SFRP4 of peak lactation mammary tissue was lower than in late lactation ($P < 0.05$), the abundance of AACS and PLIN2 was higher in peak lactation mammary compared with late lactation stage ($P < 0.05$).

To our knowledge, the differential expression of AACS, DHCR7, GSTM3, SFRP1 and SFRP4 during lactation stages were firstly detected in current study. This DEPs may play key roles in bovine lactation process. For example, milk lipids consists of triglycerides and cholesterol, which are secreted in the form of milk fat globules (MFG) [25]. AACS activates acetoacetate to acetoacetyl-CoA and the latter participates in fatty acid synthesis and cholesterol synthesis [26]. DHCR7 is a terminal enzyme of cholesterol synthesis and involved in lipid biosynthetic process and cholesterol biosynthetic process [27]. SFRP1 and SFRP4 are involved in Wnt signaling and regulate the activation of this pathway. It has been reported that SFRP4 and SFRP1 were both up-regulated at the time of apoptosis and contributed to the initiation of apoptosis in the mammary gland [28,29]. Combining our identified DEPs and milking trait related candidate genes detected in QTL mapping and GWAS studies (https://www.animalgenome.org/cgi-bin/QTLdb/BT/index), we further confirmed that proteins SFRP4 and SFRP1 associated with milk yield as well as the potential indicators of mammary epithelial cell apoptosis. Besides, nine functional well-studies known proteins (PLIN2, LPIN1, PLIN3, GSN, CD74, MMP2, SOD1, SOD3 and GPX3) were also identified differential expressed in the two lactation stages. These proteins have previously been revealed participating in lactation process [30–32]. Interestingly, CD74 molecule (CD74), matrix metalloproteinases 2 (MMP2), glutathione peroxidase 3 (GPX3) and superoxide dismutase 1 (SOD1) and superoxide dismutase 3 (SOD3) were related to immune, tissue remodeling and oxidative stress, respectively. They were all up-regulated during late lactation stage, which also be confirmed by previous bovine studies during different lactation stage [12,33]. Possible reason is that bovine mammary gland prepares for involution and the mammary gland is in proinflammatory state and becomes much more resistant to bacterial [34]. It is reasonable to expect that the up-regulation proteins related to immune functions, antioxidant may contribute to protecting against oxidative stress damage occurring in the mammary gland of dairy cows. In all, the DEPs exploited herein can be considered as milk performance related functional genes.

It is notable that the limited number of experimental animals were employed in current study which maybe weaken the power of DEP detections, and we will further validate our results in a follow-up repetition study using samples with a larger size. In addition, mutations in important DEPs should be screened and further association analysis should be performed to identify functionally important mutations and to provide more important molecular makers for cow breeding.

In summary, we identified 3753 proteins out of which 179 proteins differentially expressed during peak and late lactation stage using TMT method. We revealed five novel DEPs as well as nine known proteins related to milk performance and mammary structure. Our findings will facilitate the understanding of lactation process and provide a valuable resource for future bovine proteome studies. Taken together, the samples in the present study were normal and with no particular phenotype, these data would be a useful complement for other studies focused on the analysis of the bovine mammary gland proteome research.

Competing interests

The authors have declared that they have no competing interest.

Funding

This work was supported by the National Major Development Program of Transgenic Breeding (2014ZX0800953B); the National Natural Science Foundations of China (31272419); the Program for Changjiang Scholar and Innovation Research Team in University (IRT_15R62).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2017.10.038.

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Please cite this article in press as: X. Zheng, et al., Quantitative proteome analysis of bovine mammary gland reveals protein dynamic changes involved in peak and late lactation stages, Biochemical and Biophysical Research Communications (2017), https://doi.org/10.1016/j.bbrc.2017.10.038