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Short communication: Comparative proteomic analysis of the lactating and nonlactating bovine mammary gland

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ABSTRACT

The objective of this study was to determine how bovine mammary protein profiles vary during lactation and the dry period. Three lactating and 3 nonlactating cows were selected for mammary gland tissue sampling. Compared with the mammary proteins in nonlactating cows, a total of 60 differentially expressed proteins (DEP, including 57 upregulated and 3 downregulated) were identified in lactating cows using 2-dimensional difference gel electrophoresis combined with mass spectrometry. These DEP included enzymes and proteins associated with various macromolecular metabolic processes, and appeared to promote the increased metabolic activity associated with milk synthesis and secretion. The increased DEP were primarily related to initiation, maintenance, and involution of lactation, and included proteins involved in glycolysis/gluconeogenesis, the tricarboxylic acid cycle, the pentose phosphate pathway, oxidative phosphorylation, aminoacyl-transfer RNA biosynthesis, and fatty acid biosynthesis. Identified DEP were further validated by real-time, reverse-transcription PCR and Western blot. Five new DEP associated with lactation were uniquely identified. This work provided some protein-associated insights to facilitate further investigation of the mechanisms underlying lactation in dairy cows.
Key words: lactation, bovine mammary gland, 2-dimensional proteomics

Short Communication

The bovine mammary gland (**MG**) is a specialized organ designed to synthesize and secrete large quantities of milk (Jena et al., 2015). The bovine MG is also characterized by its successive pregnancy-lactation involution cycle (Desrivieres et al., 2007), during which the functional development of MG is hormonally regulated (Bellmann, 1976) and involves complex shifts in proteins (Jena et al., 2015). The MG of dry cows provides a baseline for the expression of enzymes and proteins at the onset of lactation (Hurley, 1989). During late gestation, the MG prepares for lactation by responding to prolactin signaling (Bellmann, 1976), the pathway of which then induces milk protein gene and biosynthetic enzyme expression to promote lactogenesis and lactation maintenance (Yang et al., 2000). Numerous early studies focused on the molecular dynamics of the MG under differing genetic, physiologic, and morphologic conditions. These studies have focused on protein and lipid synthesis during the lactation cycle (Bionaz and Loor, 2008, 2011), transcriptional differences associated with development and lactation (Li et al., 2012, 2016; Suarez-Vega et al., 2016), and immunological changes associated with mastitis (Huang et al., 2014; Zhao et al., 2015). Several groups have studied the proteomic profile of large ruminant MG during lactation, investigating shifts in carbohydrate and lipid metabolism during lactation in *Bos taurus* (Beddek et al., 2008), metabolic shifts in the MG associated with stage of lactation (Rawson et al., 2012), and MG profiles of buffaloes during lactation and the dry period (Jena et al., 2015). However, alterations in proteins related to lactation, as identified by comparing lactating and nonlactating bovine MG, have not been studied extensively with proteomic approaches. Therefore, the aim of this study was to screen for the differentially expressed proteins (**DEP**) associated with lactation in the bovine MG.

Experimental procedures were approved by the Institutional Animal Care and Use Committee at Zhejiang University, Zhejiang, China. Six multiparous and healthy, mastitis-free, Chinese Holstein cows housed at the Hangjiang Dairy Farm (Hangzhou, China) were selected for the study. Three cows in dry period (52 ± 7 mo of age; mean ± SD) and 3 cows in mid-lactation (58 ± 7 mo of age; 92 to 118 DIM) were slaughtered as described in a previous study (Dufour and Roy, 1985). Samples of 6 MG tissues were collected immediately

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after slaughter following the methods described by Wang et al. (2016). The 50-mg aliquots of MG tissue were homogenized in 0.25 mL of lysis buffer for 1 h on ice as described in Wu et al. (2012). The homogenates were centrifuged at 13,000 × *g* for 5 min at 4°C, the supernatants were then removed and proteins were collected. Protein concentrations were determined using the Bradford method (Bradford, 1976). Each 200 μg of MG proteins derived from individual samples were resolved in individual 2-dimensional (**2-D**) gels (n = 3, gels derived from 3 individual MG of 3 lactating and nonlactating groups, respectively). The procedure for 2-D electrophoresis was performed as described by Wu et al. (2012). Image Master 2D Platinum (GE Healthcare, Pittsburgh, PA) software was used for matching and analysis of protein spots in 2-D gels. Prior to analysis, all gel images were cropped to identical sizes by removing areas extraneous to the proteins spots using PDQuest 2D analysis software (version 8.0, Bio-Rad, Hercules, CA). Only the spots that were present in all gels and those that were absent from a maximum of one analytical gel per group were considered for the statistical comparison. Differential intensity levels of corresponding protein spots in the 2 treatments were analyzed by unpaired Student's *t*-test. Only the significantly expressed protein spots ($P < 0.05$) with a 2.0-fold change or more in intensity were selected for MS identification as described by Wu et al. (2012).

The gene primers (Supplemental Table S1; https://doi.org/10.3168/jds.2016-12366) were designed using DNAStar software (DNASTAR, Madison, WI). Total RNA was isolated from each MG sample using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and then reverse transcribed to cDNA using a SYBR PrimeScript RT-PCR Kit (Takara, Tokyo, Japan). The mRNA abundance of these genes was analyzed by quantitative real-time PCR (**qRT-PCR**) using SYBR Green as described previously (Zhao et al., 2010). The relative mRNA abundance was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) and normalized to β-actin mRNA in the same sample. Three replicates were performed in each sample.

The Western analysis of MG proteins [eukaryotic translation initiation factor 2 subunit 1 (**EIF2S1**), kappa-casein (**CSN3**), and β-actin] were performed as previously described in Yang et al. (2015). All the primary antibodies were purchased from Abcam (Cambridge, MA), validated with bovine samples before use, and diluted at 1:1,000 in PBS. The relative quantity of protein bands was determined by ImagePro Plus software (Media Cybernetics, Rockville, MD) and normalized to β-actin protein in the same sample. The normalized data of qRT-PCR and Western blot were analyzed by Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

The stage of lactation is one of the key drivers of milk production and milk component yields (McManaman and Neville, 2003), and a dry period of sufficient length (about 60 d) is essential for mammary cell turnover to optimize milk production following lactation (Collier et al., 2012). The mammary physiology and metabolism undergo a wide range of dynamic changes as the animal transitions from the nonlactating to lactating states (Hurley, 1989). Therefore, it is important to identify key protein alterations and molecular mechanisms underlying this transition. Compared with nonlactating bovine MG, we detected 80 DEP spots corresponded to 60 unique proteins (Supplemental Table S2; https://doi.org/10.3168/jds.2016-12366) that were differentially abundant in the lactating group. Of these proteins, 57 were upregulated (Supplemental Figure S1A; https://doi.org/10.3168/jds.2016-12366) and 3 were downregulated (Supplemental Figure S1B; https://doi.org/10.3168/jds.2016-12366). More than half of these DEP were enzymes functioning in AA, protein, lipid, and nucleotide metabolism, and their differential expression was consistent with previous studies of the lactating bovine MG (Rudolph et al., 2007; Bionaz and Loor, 2011; Rawson et al., 2012). The milk proteins α_{S2}-casein and CSN3 were significantly increased in the lactating MG; however, other casein proteins (such as α_{S1}-isoforms and β-isoforms) were not observed. Variation in 2-D proteomic analysis via gel exercising followed by MS identification may account for the absence of these casein isoforms.

Some DEP that have not been reported before in the MG during lactation were detected, including mitochondrial inner membrane protein (**IMMT**), 3-hydroxyisobutyrate dehydrogenase (**HIBADH**), dimethylarginine dimethylaminohydrolase 1 (**DDAH1**), *N*-acetylneuraminic acid synthase (**NANS**), endoplasmic reticulum lectin 1 (**ERLEC1**), and sec13 protein homolog (**SEC13**). The protein DDAH1 is an enzyme that metabolizes methylated arginine to citrulline and methylamine, which regulates organ development (in brain, kidney, and gastrointestinal tissues) in adult rats and chickens (Mishima et al., 2004; Breckenridge et al., 2010). Although its definite function in mammary development has not been elucidated, the upregulation of DDAH1 ($P = 0.0011$) within the lactating MG might be related to mammary differentiation. Also, the increased protein SEC13 is mainly involved in the biogenesis of coat protein complex II (**COPII**)-coated vesicles and protein transport (Enninga et al., 2003) and acts as 1 of the 5 components in GTPase-activating protein target of rapamycin 2 complex, activating the