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Crucial genes at the onset of lactation revealed by transcriptome screening of domestic yak mammary gland

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ABSTRACT

At the onset of lactation, there are three distinct stages of mammary tissue development and function including mammogenesis, colostrogenesis, and lactogenesis. The mechanism of the transition from colostrogenesis to lactogenesis of Maiwa Yak is still unknown. In this study, mammary tissues from three Maiwa yaks were collected at 1 and 30 d after parturition for transcriptome exploration using Affymetrix Bovine Genome Arrays. Comparing 1 and 30 d results, a total of 517 annotated differentially expressed genes (**DEG**) were identified at the criteria of a p-value ≤ 0.05 . The ratio of up-regulated genes to the down-regulated ones was around 1:2 (more specifically, 164:353). To depict the profile of DEG, a Dynamic Impact Approach (**DIA**) was used to analyse the microarray data based on Gene Ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) databases. GO terms 'fatty acid transport' and 'monocarboxylic acid transport' were significantly induced during the colostrum period. The strongly impacted KEGG pathways were 'Chondroitin sulfate biosynthesis', 'Glycosphingolipid biosynthesis' and 'Glycerolipid metabolism'. These data may provide candidate genes with a high probability of having functional roles in regulating the transition from colostrum to normal milk in domestic yak mammary gland.

Key Words: Maiwa yak, mammary gland, milk component, transcriptome, gene expression profile

Short Title: Domestic Yak Mammary Gland Transcriptome**INTRODUCTION**

Due to conditions of extreme harshness, including extremely cold, low pressure and low oxygen content of the air with high solar radiation at high altitude in the Tibetan Plateau, yak becomes the main source of the milk, meat and other necessities for Tibetan (Qiu et al. 2012; Weiner et al. 2003). As for yak milk, it's also the raw material of other local residents' traditional food, such as milk tea, butter, milk residue and so on. Five stages exist in the process of mammary gland development, of which colostrogenesis, lactogenesis and lactation are related to the conditions of the mammary gland in the colostrum and normal milk period (Barrington et al. 2001). In bovids, colostrum is the milk in the first 4 days postpartum; milk produced during the rest of the lactation constitutes normal milk (Gopal and Gill 2000). As complete sources of nutrients and initial acquired immunity for neonates, colostrum and normal milk have their special values for the growth of newborn calves (Cui et al. 2014; Stelwagen et al. 2009). But their gross compositions, physical properties and suitability for processing are much different in *Bos* genus (Cui et al. 2014; Stelwagen et al. 2009; Tsioulpas et al. 2007). The concentration of immunoglobulins drops from 47.60, 2.90, 3.90 and 4.20 mg/ml for IgG1, IgG2, IgA and IgM separately in colostrum to 0.59, 0.02, 0.14 and 0.05 mg/ml in normal milk (Stelwagen et al. 2009). The mean concentration of colostral

immunoglobulin G of dairy cows decreases rapidly (113, 94, 82, and 76 g/L at 2, 6, 10, and 14 hours postpartum) in the first 24 hours after calving (Moore et al. 2005). Especially, the extraordinary high value of IgG1 in the colostrum is caused by the selective transport of it into colostrum at the colostrogenesis stage (Barrington et al. 2001).

Almost all the milk compositions decrease markedly in the first 3 days postpartum, while the lactose increases (Cui et al. 2014). The percentage of milk solid in colostrum is almost twice that of the normal milk. Besides, the fat and protein can reach as high as almost three-fold that of the normal milk (Weiner et al. 2003). In the study of Tsioulpas et al. (Tsioulpas et al. 2007), higher Ca, free ionic Ca, P, Mg, and Na were observed during the colostrum period compared with the normal milk period. Lower pH, ethanol stability and higher free ionic calcium concentration result in the very low stability of yak milk on the first day postpartum. The stable decrease from day 1 to day 5 in ionized Ca also results in a progressive increase in ethanol stability (Tsioulpas et al. 2007).

Although so many advantages of colostrum are known by now, the ways by which the formation of colostrum are regulated in mammary gland remains unclear. The objective of our study is to understand how gene expression changes between the colostrum and normal milk period in the domestic yak's mammary gland. An Affymetrix Bovine Genome Array was used to determine the differentially expressed genes (**DEG**) in yak mammary glands between the colostrum period and the normal milk period, and then a Dynamic Impact Approach

(DIA) method was used to depict the potential mechanism under these data. Our results may provide candidate genes with high probabilities of having functional roles in regulating transition from colostrum to normal milk.

MATERIAL AND METHODS

Animals and Sampling

The Animal Care and Use Committee of the Southwest University for Nationalities approved all procedures and experiments (2013-2-1). Three healthy adult Maiwa yaks with similar weight, age and production characteristics were selected from the Hongyuan county of the Sichuan province (around 102.55°E, 32.80°N, ~3490 m above sea level, from Google map). Surgery was performed by a veterinarian, and approximately 1g of tissue was obtained at day 1 and day 30 postpartum from the middle area of mammary glands, respectively. After getting rid of the fat tissues and connective tissues, the relative pure mammary gland tissues were washed by diethyl pyrocarbonate treated water, cut into small pieces, and immediately stored in liquid nitrogen for total RNA extraction.

Total RNA Isolation, Purification and Quality Control

Tissue RNA was extracted using TRIZOL reagent (Invitrogen Life Technologies, CA, US) following the manufacturer's instruction, which was then purified using RNeasy Kit

(QIAGEN, GmbH, Germany). A RIN number of 7.0 was used to guarantee a higher RNA integrity by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US). Qualified total RNA was further purified by RNeasy micro kit (Cat. #74004, QIAGEN, GmbH, Germany) and RNase-Free DNase Set (Cat. #79254, QIAGEN, GmbH, Germany).

Microarray Hybridization and Analysis

Array Hybridization and washes were performed according to the manufacturer's instructions of GeneChip® Hybridization, Wash and Stain Kit (Cat. #900720; Affymetrix, Santa Clara, CA, US) in Hybridization Oven 645 (Cat. #00-0331-220V; Affymetrix, Santa Clara, CA, US) and Fluidics Station 450 (Cat. #00-0079; Affymetrix, Santa Clara, CA, US). The analysis of domestic yak mammary transcriptome at colostrum and normal milk periods was done using the GeneChip Bovine Genome Array (Affymetrix, Santa Clara, CA, USA), which contains 24,072 probe sets representing more than 23,000 transcripts and includes approximately 19000 UniGene clusters.

Arrays were scanned by GeneChip Scanner 3000 (Cat. #00-0012, Affymetrix, Santa Clara, CA, US) with default settings. Raw data were normalized by MAS 5.0 algorithm, and GeneSpring Software 11.0 (Agilent technologies, Santa Clara, CA, US). Differentially expressed genes were screened under the criteria of $P\text{-value} \leq 0.05$. The Bovine 3'-IVT Expression Array CSV (comma-separated values) annotation file (Release 36, published on

April 13, 2016) obtained from NetAffx Analysis Center was used to annotate our bovine 3'-IVT expression microarray for more information about each probe. Then the DEGs list was uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al. 2009) website for enrichment analysis and the significant enrichment biological terms of the selected annotation databases (Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and some other databases we were interested in) were found by the tool 'Functional Annotation' provided in the DAVID website. EASE score = 1 and Count = 2 were set in the 'Functional Annotation Chart' so we could get the whole results of enriched biological terms which were hit by just 2 genes.

Because enrichment analysis can only tell us the biological terms which are significantly enriched, how much these terms are impacted by the treatment and the direction of the impact are still unknown according to this method. So a more powerful method DIA (Bionaz et al. 2012a; Bionaz et al. 2012b) was chosen to measure the impact and the direction of each term. Fold changes, p-values and the proportion of genes which are differentially expressed compared to all the genes in one biological term are taken into account in the calculation of the impact value and the direction value of this term. It takes almost all the data that is pertinent to the biological terms into account and finally provides researchers with a measurement about how much the specific biological term impacts the treatment and the direction of the impact those researchers are interested in.

For a specific biological term, its impact and flux (direction of the impact) are calculated as follow: $\text{Impact} = 1 / b \times (a \times c \times d + e \times f \times g)$ (Eq. 1); $\text{Flux} = 1 / b \times (a \times c \times d - e \times f \times g)$ (Eq. 2). (a: No. of up regulated DEGs in a specific term; b: Total no. of genes in the term present in our array; c: Average $\log_2$ (Fold change of up regulated gene members in a term); d: Average $-\log_{10}$ (P-value of up regulated gene members in a term); e: No. of down regulated DEGs in a specific term; f: Average $\log_2$ (Fold change of down regulated gene members in a term); g: Average $-\log_{10}$ (P-value of down regulated gene members in a term)).

And the procedure of the whole calculation was implemented through our home-made Perl scripts. In our scripts, the genes were mapped to biological terms by DAVID-WS (Jiao et al. 2012). Then the impact value and flux value of each term were calculated according to the formulas mentioned above.

RESULTS AND DISCUSSION

There are 12 breeds of domestic yaks that are officially recognized (Weiner et al. 2003). In our research, we chose one representative type, the grassland Maiwa yak of Sichuan, because of its milk production relative to other breeds. The samples of mammary tissues were taken from the same 3 yaks at two time points with themselves as the control group. So the error which would be introduced by using different individuals at 2 time points was avoided and eliminated out of statistics analysis. Also it weakened the influence of our relative small

sample size. The data shown here would be informative and reliable for the study on lactation regulation mechanism in yak despite the lack of validation using qRT-PCR. More yak mammary gland tissue samples in different lactation periods will be collected and used to identify the profiles of the DEGs in our lab. At the criteria of the P-value ≤ 0.05 , 636 genes were identified differentially expressed in the colostrum period with the normal milk period as the control of which 517 were well annotated and used in the downstream analysis. All analyses and comparisons in our experiments used the observations at 30 d as the control.

All 517 DEGs with well-annotated information are contained in the Supplementary Material 1 and all enriched biological terms (like GO terms and KEGG pathways) can be found in Supplementary Material 2. The DEGs of each enriched biological term were also appended to the end of each term in Supplementary Material 2 to facilitate exploring genes contributing to the identification of the terms.

The details of top 6 most impacted GO terms (including top 3 up-regulated terms and top 3 down-regulated terms) in each of 3 GO categories are shown in Fig. 1. The top 30 most impacted KEGG pathways (including top 15 up-regulated pathways and 15 down-regulated pathways) are shown in Fig.2. Considering the object we studied, the category 'Human Disease' was removed from the results of KEGG enrichment analysis. The up-regulated GO terms or KEGG pathways were sorted by the value of their impacts in descending order and the down-regulated ones were in ascending order.

GO Analysis

Among the top 6 most impacted GO terms, ‘fatty acid transport’, ‘protein serine/threonine phosphatase complex’ and ‘ribosome binding’ were highly activated and ‘hormone binding’, ‘positive regulation of tissue remodelling’ and ‘synaptic vesicle’ were significantly inhibited.

Considering around 25% to 65% of milk fat comes directly from dietary fat (Glascock et al. 1966; Palmquist and Conrad 1971), a stronger fatty acid transport process might be beneficial for improving the milk fat content in bovine milk. The up-regulation of this term, which was caused by the up-regulated gene *SLC02A1* (solute carrier organic anion transporter family, member 2A1, P-value = 0.006274 and Fold-Change = 4.832891) and *ANXA1* (annexin A1, P-value = 0.013639 and Fold-Change = 2111394), was also consistent with the reported research results. Fat content decreased rapidly in the first 7 days postpartum from 7.89 g/100g (milk) at day 1 postpartum to 5.85 g/100g (milk) at day 7 postpartum (Cui et al. 2014). Thus, although more specific experiments are still needed to explore the procedure of the formation of colostrum in the future, the highly induced ‘fatty acid transport’ in the colostrum period may partly explain the higher fat content in yak colostrum.

Another highly impacted term captured due to the same genes with the term ‘Fatty acid transport’ was ‘Monocarboxylic acid transport’. The short- and medium-chain saturated fatty acids, like ethanoic (esterified to glycerol in ruminant milk fats), butanoic (in milk fat of

ruminant), hexanoic (in milk fat), octanoic (major component of milk) and decanoic (major acid in milk) (Guschina and Harwood 2013), are monocarboxylic acid (or monocarboxylate) (Halestrap 2013). Nine monocarboxylate transporter genes (**MCT**) isoforms (MCT1, 2, 3, 4, 5, 8, 10, 13 and 14) are found to exist in the mammary gland of lactating cattle (Kirat and Kato 2009). MCT1 plays an important role in absorbing short-chain fatty acids like butyrate and propionate from the gut into the blood (Lamers and Hülsmann 1975; Lamers and Kurpershoek-Davidov 1975; Ritzhaupt et al. 1998). MCTs 1-4 play important roles in intestinal metabolism of short-chain fatty acids with proton-linked monocarboxylate transport (Halestrap and Meredith 2004; Halestrap and Wilson 2012). It seems that the high induction of monocarboxylic acid transport in the present study might be caused by the demand for monocarboxylate.

KEGG Analysis

The rationale of KEGG analysis is to assign different genes to different KEGG pathways and find out the significantly-enriched pathways, which aims at helping researchers identify biological processes most pertinent to their studies (Kanehisa et al. 2015). The significantly enriched pathways were mainly contained in the categories 'Lipid metabolism' (6 pathways), 'Signal transduction' (6 pathways) and 'Endocrine System' (6 pathways), which accounted for

one third (18 pathways) of the total number (59 pathways) of our significantly enriched pathways.

The 'Chondroitin sulfate biosynthesis' was the most impacted pathway (caused by *DSE* (dermatan sulfate epimerase, P-value = 0.026941 and Fold-Change = 3.679741) and *CHSY1* (chondroitin sulfate synthase 1, P-value = 0.046265 and Fold-Change = 2.359144)) at day 1 relative to day 30 (Fig. 2.). The DIA uncovered a significant synthesis stimulation of chondroitin sulfate, a kind of sulfated glycosaminoglycans and also one component of the cell surface proteoglycans of the NMuMG mouse mammary epithelial cells (Rapraeger et al. 1985). Although the exact role the chondroitin sulfate in regulating the development of the bovine mammary gland has not yet been reported, the absence of chondroitin sulfate chains in the syndecan-1 and syndecan-4 from normal murine mammary gland epithelial cells would lead to the change of their kinetics of binding to midkine, pleiotrophin, and basic fibroblast growth factor (Deepa et al. 2004). In addition, there are differences in the structure and the function of chondroitin sulfate chains. The synergetic effects of various chondroitin sulfate chains and heparin sulfate chains could facilitate the binding of syndecans, which are receptors for soluble ligands (Braun et al. 2012) and expressed in almost all epithelial cells as the major cell surface proteoglycans (Deepa et al. 2004), with much more different growth factors. From earlier studies, it is known that bFGF acts as growth proliferative, differentiation and chemotactic factors (Coleman-Krnacik and Rosen 1994) and

mammogenesis doesn't totally end postpartum (Richert et al. 2000) and continues into the early stage of lactation (Sordillo et al. 1984). The relative up-regulation of 'chondroitin sulfate biosynthesis' during the colostrum period might be one of the reasons for this phenomenon.

The present study also had a novel finding, the inhibition of 'Glycosphingolipid biosynthesis' (caused by **B3GNT3** (UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 3, P-value = 0.03544 and Fold-Change = 0.497158) and **ABO** (ABO blood group (transferase A, alpha 1-3-N-acetylgalactosaminyltransferase; transferase B, alpha 1-3-galactosyltransferase), P-value = 0.006635 and Fold-Change = 0.424809)). Glycosphingolipid is a subtype of glycolipid and one class of sphingolipids which constitutes 'lipid rafts', a kind of small lateral microdomains made up with a mass of lipid molecules and a few protein molecules (Korade and Kenworthy 2008; Thomas et al. 2004a; Thomas et al. 2004b). Several important receptors are localized in lipid rafts microdomains, such as growth factor receptors, T cell receptors and insulin receptor (Bromley et al. 2001; Paratcha and Ibanez 2002). Considering the modulating capability of glycosphingolipid to the signaling functions mentioned above, on the basis of the known epidermal growth factor and insulin's effect on mammary gland development (Tonelli and Sorof 1980) and milk secretion (Prosser et al. 1990), it could be inferred that cell signal transduction concerning mammary gland development could be intermediated by glycosphingolipid to a certain extent (Schnaar et al.

2009). Gangliosides are one kind of glycosphingolipid in which the membrane surrounding fat globules in milk are enriched (Keenan 1974; Keenan et al. 1972). The inhibition of 'Glycosphingolipid biosynthesis' here was consistent with the change of the amount of the gangliosides in the milk.

The fat content in colostrum of yak is significantly higher than that in the normal milk (Cui et al. 2014; Sarkar et al. 1999; Weiner et al. 2003). Glycerolipid, which is best-known as the fatty acid triesters of glycerol, called triglycerides (also known as fat), is a heterogeneous group of lipids, so it's not surprising that they may share some common metabolites. The significant inhibition of 'Glycerolipid metabolism' (Fig. 2.) according to the DIA analysis might be due to the significant down-regulation of these genes that are associated with glycerolipid catabolism. They are **CEL** (carboxyl ester lipase, P-value = 0.044944 and Fold-Change = 0.576049), **PNLIP** (pancreatic lipase, P-value = 0.031043 and Fold-Change = 0.157440) and **DGKH** (diacylglycerol kinase, delta 130kDa, P-value = 0.02409 and Fold-Change = 0.459998). As the main source of milk fat, triacylglycerol is made up of fatty acid chains and glycerol (Bauman and Davis 2013). **CEL** is a lipolytic enzyme capable of hydrolyzing mono-, di-, and tri-acylglycerols and **PNLIP** is also the main enzyme for the hydrolysis of triacylglycerol into monoglycerides and fatty acids. The conversion of diacylglycerol to phosphatidic acid is catalyzed by **DGKH**. It could be assumed that the

down-regulation of the expression of *CEL*, *PNLIP* and *DGKH* might be the reason for the high content of fat in the colostrum (Fig. 3.).

Because of the lack of a yak-specific microarray, the cattle microarray was used instead. Lots of genetic characters were shared by them, like both having 30 chromosomes and karyotypes (Weiner et al. 2003). Between yak and cattle, 45% of encoded proteins were totally identical and mean protein similarity reached around 99.5% according to a recent genomic sequence and analysis of a female domestic yak (Qiu et al. 2012). Because no yak microarray was available, it was reasonable to use the cattle microarray to inspect the transcriptome of the yak. Although we were unable to decipher which genes were found exclusively in yak (and not in cattle), it was reasonable to use the cattle microarray to inspect the transcriptome of yak. Efficiency of the microarray was impaired because some probes were specific to cattle.

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LEGENDS

Fig. 1. The top three up- and the top three down-regulated terms of each of three categories in GO (Gene Ontology) enrichment annotation results. The 'term' column illustrated GO terms. The horizontal blue bar in the 'impact' column showed the intensity of impact of the differentially expressed genes on the go term (Longer the horizontal bar larger the impact). In the 'flux' column, the red bar denoted induction of the term and the green bar denoted inhibition (Darker the color larger the intensity of induction or inhibition).

Fig. 2. The top 15 up- and the top 15 down-regulated DIA (Dynamic Impact Approach) results of the KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment pathways. Dark blue horizontal bars denoted the impact and the columns right with them denoted the direction of the impact (the color red means induction and the green means inhibition).

Fig. 3. Visualization of the 'Glycerolipid metabolism' pathway. The nodes in this pathway which belong to *Bos taurus* were noted with green and those comprising the differentially expressed genes were noted with red. Especially, the DEGs were labelled manually alongside the nodes comprising them.

Fig. 1.

Category	GO Term	Impact	Flux
Molecular Function	GO:0004690~cyclic nucleotide-dependent protein kinase activity	236.577	236.577
	GO:0005338~nucleotide-sugar transmembrane transporter activity	54.897	54.897
	GO:0043022~ribosome binding	47.977	47.977
	GO:0042562~hormone binding	99.890	-99.890
	GO:0042301~phosphate binding	123.749	-123.749
	GO:0004977~melanocortin receptor activity	403.965	-403.965
Biological Process	GO:0046824~positive regulation of nucleocytoplasmic transport	183.034	183.034
	GO:0015908~fatty acid transport	85.195	85.195
	GO:0032388~positive regulation of intracellular transport	78.443	78.443
	GO:0046449~creatinine metabolic process	288.748	-288.748
	GO:0006105~succinate metabolic process	288.748	-288.748
	GO:0034105~positive regulation of tissue remodeling	426.035	-426.035
Cellular Component	GO:0008287~protein serine/threonine phosphatase complex	47.970	47.970
	GO:0030018~Z disc	40.221	40.221
	GO:0031674~I band	36.564	36.564
	GO:0044456~synapse part	24.945	-0.109
	GO:0045202~synapse	25.517	-0.167
	GO:0008021~synaptic vesicle	30.065	-30.065

Fig. 2.

KEGG Pathway	Impact	Flux
Chondroitin sulfate biosynthesis	34.828	34.828
Olfactory transduction	22.531	22.531
Ubiquitin mediated proteolysis	21.247	1.038
Ether lipid metabolism	20.859	20.859
Toll-like receptor signaling pathway	20.060	12.773
Gap junction	17.592	17.592
Melanogenesis	15.450	4.415
GnRH signaling pathway	14.099	14.099
MAPK signaling pathway	13.161	6.431
Wnt signaling pathway	12.583	2.822
Adipocytokine signaling pathway	12.226	12.226
Adherens junction	11.164	11.164
Cell cycle	10.469	10.469
Spliceosome	8.553	5.283
Progesterone-mediated oocyte maturation	7.705	7.705
Tight junction	12.585	-0.884
Notch signaling pathway	13.334	-13.334
Steroid biosynthesis	13.337	-13.337
Arachidonic acid metabolism	15.201	-15.201
Retinol metabolism	15.781	-15.781
p53 signaling pathway	16.955	-16.955
N-Glycan biosynthesis	17.519	-7.239
Drug metabolism	17.622	-17.622
Glycosphingolipid biosynthesis	18.045	-18.045
Purine metabolism	18.721	-18.721
Glycerolipid metabolism	20.710	-20.710
Metabolism of xenobiotics by cytochrome P450	20.731	-20.731
Other glycan degradation	21.430	-21.430
Dorso-ventral axis formation	29.954	-29.954
Neuroactive ligand-receptor interaction	30.064	-18.557

[illegible]