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Identification and verification of differentially expressed genes in yak mammary tissue during the lactation cycle

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Research Article

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Abstract

Yaks (*Bos grunniens*) live primarily in the Qinghai-Tibetan plateau (altitude: 2000–5000 m). Their milk presents unusual characteristics, containing large amounts of solids including fat and protein, and it is, therefore, important to understand the genetic makeup of the yak. To identify potentially critical genes playing a role in yak mammary tissue from colostrum to mature milk phase of lactogenesis, the early lactation (colostrum) stage (ELS; day 1 after parturition) and mature lactation (milk) stage (MLS; day 15) were chosen for comparison. An ELS-specific cDNA library was established by suppression subtractive hybridization and 25 expressed sequence tags at ELS were identified by sequencing and alignment. To further confirm our results the expression levels of 21 genes during the lactation cycle were measured using quantitative real-time RT-PCR (qRT-PCR). The qRT-PCR results confirmed 9 significantly up-regulated genes at ELS vs. MLS in yak mammary tissue, in which the L-amino acid oxidase 1 (*LAO1*) and collagen, type I, alpha I (*COL1A1*) were the most significantly up-regulated. During the lactation cycle, the highest expression of some milk fat genes (i.e., *XDH* and *FABP3*) in yak mammary tissue appears earlier than that in dairy cow. Our data also indicate *MYC* potentially playing a central role through putative regulation of *COL1A1*, *CD44*, *SPARC*, *FASN* and *GPAM*.

More than 14 million yak are living in the Qinghai-Tibetan plateau, whose milk contribute 80% of gross milk production in this area (Wiener *et al*., 2006). The yak has adapted to thrive under these harsh conditions, where few others domestic animals could survive. The yak is a crucial resource supplying milk, meat, hair, and cheese to the local people in the Tibetan plateau area. For these reasons, the yak is one of the most critical domestic animals for 6.5 million Tibetan people (He and Li, 2004). Based on the fact that yak represent the primary source of milk for the people of this region, it is important to understand the gene expression pattern of yak mammary tissue.

Colostrum immunoglobulin G (IgG) is of major importance for the newborn calf because epitheliochorial placentae do not provide transfer of immunoglobulins utero. Calves are highly dependent on the first milk (colostrum), which contains the important immunoglobulins and a high content of proteins and fat (Weaver *et al*., 2000). Colostrum is secreted during a colostrogenesis stage. Compared to milk, colostrum has higher concentration of immunoglobulins, specifically immunoglobulin G1 (Dang *et al*., 2009) as well as other plasma-derived and secreted proteins. Study of genes that are differentially expressed during this period has not been done, and was the objective of our research. We compared 1 d (ELS) and 15 d postpartum (MLS) to identify the specifically up-regulated genes through the construction of suppression subtractive hybridization (SSH) library. Thereafter the differentially expressed genes were verified by qRT-PCR during the entire lactation period.

Materials and methods

Mammary tissue collection

This study was approved by the Southwest University for Nationalities Institutional Animal Care and Use Committee (permit number: 2011-3-2). The yak mammary biopsy was performed in strict accordance with the animal operations guide to minimize animal suffering. Tissue samples of yak mammary gland were collected from 4 healthy female yaks (approximately 5 years old) in the northwest plateau of Sichuan province in China.

Percutaneous biopsies were obtained alternatively from the right or left rear quarter of the mammary gland at −15, 1, 15, 30, 60, 120, 240 d following a previously developed technique (Knight *et al*., 1992). Briefly, blunt dissection of the mammary capsule was performed to

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obtain mammary parenchyma after making the skin incision. The wound was closed with catgut sutures. The incision site was sprayed with topical antiseptic (Povidone Iodine Ointment, 10%; Taro Pharmaceuticals, China). A total of approx. 300 mg tissue sample was placed into a 5 ml tube and immediately frozen and stored in liquid nitrogen.

Extraction of total RNA and preparation of cDNA

Total RNA was extracted with ice-cold Trizol reagent (Invitrogen, USA) following the manufacturer's instruction. Each RNA sample was treated with DNase I (TaKaRa, Japan) and then dissolved in RNase-free water before storage at −80°C. The purity and concentration of RNA samples was detected by ultraviolet spectrophotometer. The 260/280 ratios of the extracted RNA samples were ≥1.9 and the integrity of the RNA samples was evaluated by 1% gel electrophoresis. Two expected bands at 18S and 28S without any evidence of degraded products was found in all RNA samples. Poly-(A) RNA was purified from total RNA using the Oligo-tex-dT mRNA Midi Kit (Qiagen, Germany). The double-stranded-cDNA of each sample was synthesized *via* PCR cDNA synthesis Kit (TaKaRa, Japan) according to the manufacturer's instruction.

Subtractive cDNA library construction

Suppression subtractive hybridization was performed using the PCR-selected cDNA Subtraction Kit (TaKaRa, Japan) according to the manufacturer's instruction. The forward-subtraction starting material consisted of 0.5 µg of mRNA from 1 d as the tester and 0.5 µg mRNA from 15 d as the driver. All PCR products generated from forward- and reverse-subtraction were directly ligated into a pMD18-T vector (TaKaRa, Japan), and then transformed into *E. coli* strain JM109. Transformed *E. coli* were plated on Luria-Bertani-Ampicillin media. Recombinant clones were kept in Luria-Bertani broth containing 15% glycerol and 100 µg/ml ampicillin (TaKaRa, Japan). The synthesized cDNA ranged approximately from 250 to 4500 bp before *Rsa* I digestion and 100 to 1000 bp after digestion in gel electrophoresis. Subtractive PCR products at ELS were subsequently obtained using the SSH method.

DNA sequence analysis for the identification of gene annotation

Fifty-six clones were randomly selected from the Subtractive cDNA Library and sequenced. The expressed sequence tags (ESTs) at ELS were identified by DNAMAN software (Lynnon Corporation) and the redundancy sequences were discarded. All the identified sequences were compared with the Cow (*Bos taurus*) Genome Browser Gateway (http://genome.ucsc.edu) using the BLAT algorithm (Kent, 2002) and annotated.

qRT-PCR

Twenty-one genes were selected to detect the expression level during the lactation cycle by qRT-PCR (online Supplementary File Table S1). The cDNA was synthesized from 600 ng of total RNA using a cDNA synthesis Kit (TaKaRa, Japan). The qRT-PCR was performed using the following reaction mixture: 5 µl SsoFast EvaGreen supermix (BIO-RAD, USA), 2 µl cDNA template, 2 µl ddH_2O and 0.5 µl 10 µM of each forward and reverse

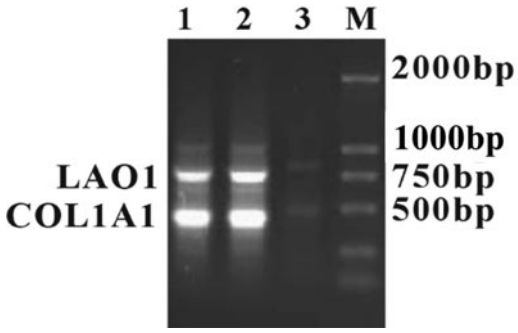

Fig. 1. Subtractive PCR products at early lactation stage. *Notes*: Lanes 1 and 2: 2nd PCR product; lane 3: 1st PCR product; lane M: marker.

primer (online Supplementary File Table S1) and the reaction was performed at 95°C for 5 min (initial denaturation) and followed by 40 cycles of at 95°C for 15 s and at 58.0°C for 30 s (amplification) in a BIO-RAD CFX96™ Real-Time System (BIO-RAD, USA). The no template control and samples were run in triplicate. Normalization was performed using 3 internal control genes (mitochondrial ribosomal protein S15, ribosomal protein S23 and ubiquitously expressed transcript isoform 2) previously determined to provide a reliable normalization (Jiang *et al.*, 2016).

Data processing and statistical analysis were conducted as described by Bionaz and Loor (Bionaz and Loor, 2008). Briefly, normalized qRT-PCR data are presented as *n*-fold change relative to −15 d before parturition. The fixed effect in the model was time (−15, 1, 15, 30, 60, 120, and 240 d) and the random effect was yak.

Statistical analysis of qPCR data

All PCR data were normalized using 3 the internal control genes (before statistical analysis; Jiang *et al.*, 2016). Data processing and statistical analysis were conducted as described by Bionaz and Loor (Bionaz and Loor, 2008). Briefly, normalized qRT-PCR data normalized data were transformed to obtain a perfect average of 1.0 at −15 d. The same proportional change was calculated at the remaining time points and is presented as n-fold change relative to −15 d before parturition. All data were log 2 transformed before statistical analysis and then analyzed using the Proc MIXED of SAS with repeated measures (v 9.4, SAS Institute Inc., Cary, USA) to evaluate the effect of time. Compound symmetry was used for analysis. The fixed effect in the model was time (−15, 1, 15, 30, 60, 120, and 240 d) relative to parturition and the random effect was caused by individual yak. The data was mentioned only if an overall time effect ($P \leq 0.05$) was present. Comparison between time points was declared significant at $P < 0.05$.

Functional enrichment and gene network analysis

David version 6.7 (https://david.ncifcrf.gov/) was used to perform functional annotations of biological processes affected by differentially expressed genes list between ELS and MLS. Gene network pathways were evaluated using Ingenuity Pathway Analysis (IPA, Ingenuity, USA). This is a web-based application that enables the discovery, visualization, and exploration of interaction networks. The software relies on currently known relationships (i.e., published manuscripts) among human, mouse, and rat genes or proteins.

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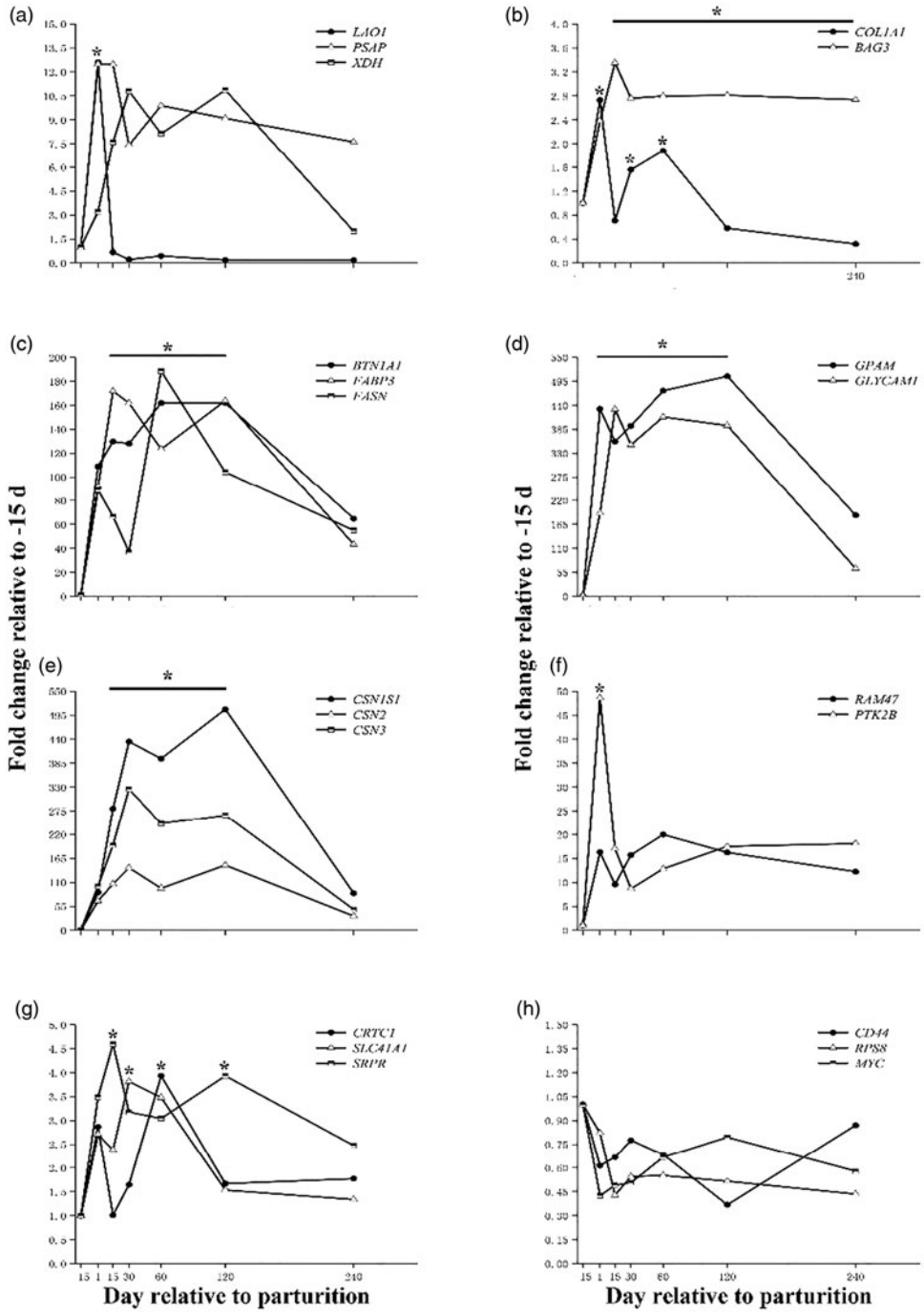

Fig. 2. Expression pattern of 21 identified genes.

Results and discussion

Suppression subtractive hybridization results for the identification of genes which are primarily expressed at ELS in comparison to MLS

Two main subtractive PCR products L-amino acid oxidase I (*LAO1*) and collagen, type I, alpha I (*COL1A1*) at ELS were discovered in gel electrophoresis (Fig. 1). *LAO1*, which has never been cloned in ruminant animals, increased from −15 to 1 d in yak mammary tissue and peaked at ELS in this study (Fig. 2a). The proteins designated *LAO1* and *LAO2* have potent antibacterial properties associated with L-amino acid oxidase activity in snake (Stiles *et al.*, 1991). Therefore, the early lactation stage probably has better antibacterial properties than mature lactation stage. *LAO* produced H_2O_2 through the oxidation of sufficient free amino acids in mouse mammary tissue and the H_2O_2 is the active component of the antibacterial system (Green *et al.*, 1944; Klebanoff *et al.*, 1966). Colostrum protects newborn calves from pathogens because it contains immunoglobulins (Barrington *et al.*, 2001). In addition, *LAO* expressed in mouse milk (Sun *et al.*,

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Table 1. Expressed sequence tags (ESTs) expressed at lactation stage and mapped to the bovine genome in GenBank

EST accession	Gene	Gene description	GenBank accession	Score	Identity (%)
FG589010	*LAO1*	*Bos taurus* similar to L-amino acid oxidase 1	XM_001251177.3	1127	98
FG588981	*FASN*	*Bos taurus* fatty acid synthase	XM_005905364.1	1024	99
FG589012	*CRTC1*	*Bos taurus* CREB regulated transcription coactivator 1	XR_239191.1	582	98
FG589013	*CD44*	*CD44* antigen	XM_005890900.1	706	99
FG589015	*RBM47*	*Bos taurus* similar to RNA-binding protein 47, transcript variant 1	XM_005207891.1	769	99
FG589018	*PSAP*	*Bos taurus* prosaposin	BC105409.1	791	99
FG588961	*SPARC*	*Bos taurus* secreted protein, acidic, cysteine-rich	BT030548.1	763	99
FG588963	*COL1A1*	*Bos taurus* collagen, type I, alpha 1	NM_001034039.2	861	100
FG588964	*SLC41A1*	*Bos taurus* solute carrier family 41, member 1, transcript variant X2	XM_005216684.1	778	99
FG589000	*BAG3*	*Bos taurus* BCL2-associated athanogene 3	NM_001082471.2	518	97
FG589001	*CSN1S1*	*Bos taurus* casein alpha s1	BC109618.1	1147	99
FG589023	*GLYCAM1*	*Bos taurus* glycosylation-dependent cell adhesion molecule 1	NM_174828.2	1040	98
FG589030	*GPAM*	*Bos taurus* mitochondrial glycerol phosphate acyltransferase	XR_240212.1	854	99
FG589032	*PTK2B*	*Bos taurus PTK2B* protein tyrosine kinase 2 beta	NM_001102252.1	743	99
FG589004	*CSN2*	*Bos taurus* casein beta	S67277.1	1005	99
FG588970	*RPS8*	*Bos taurus* ribosomal protein S8	BC102608.1	667	100
FG588974		No significant similarity found			
FG588975	*SRPR*	*Bos taurus* signal recognition particle receptor	NM_001075122.1	778	98
FG588976	*BTN1A1*	*Bos taurus* gene encoding butyrophilin	Z93323.1	743	99
FG589008	*XDH*	*Bos taurus* mRNA for xanthine oxidoreductase	NM_173972.2	1391	99
FG589009		*Bos grunniens* isolate bg008 mitochondrion, complete genome	EF494179.1	1332	99
FG589011	*FABP3*	*Bos taurus* fatty acid binding protein 3	XM_005202891.1	902	99
FG588993	*CSN3*	*Bos taurus* casein kappa	NM_174294.2	946	99
FG588980	*PACSIN2*	*Bos taurus* protein kinase C and casein kinase substrate in neurons 2	NM_001046468.1	797	88
FG588998	*MYC*	*Bos taurus* v-myc myelocytomatosis viral oncogene homolog (avian)	NM_001046074.2	479	87

EST, expressed sequence tags.

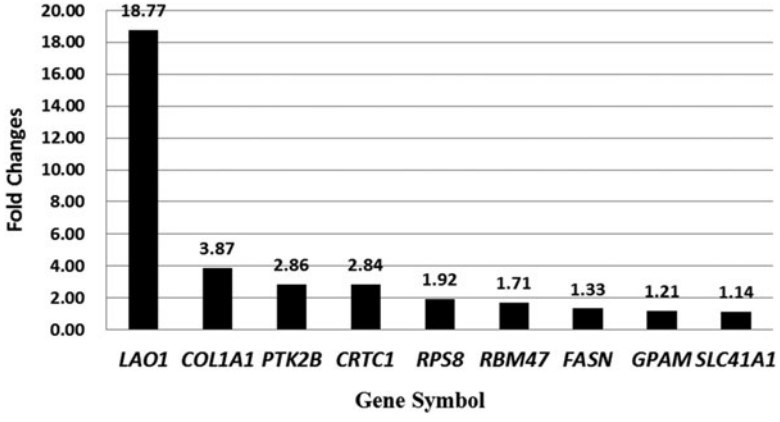

Fig. 3. Nine significantly up-regulated genes (*X*-axis) at early lactation stage relative to mature lactation stage.

2002) may also play an important role protecting the newborn. In our study, *LAO1* sharply decreased during 1 to 15 d in yak mammary tissue and maintained a low expression level after 15 d. Previous research (Nagaoka *et al*., 2014) suggested that mammary gland in dairy cows expressed low level of the *LAO* compared with that in mice, which may results in a high risk of mastitis.

COL1A1 is one of the main components of the extracellular matrix of mammary tissue and is believed to be involved in

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Table 2. Functional classification of the genes potentially associated with in Yak mammary tissue during the early lactation stage

	Term	Gene count	*P*-value	Specific gene enriched
1	Animal organ development	11	8.2×10^{-5}	XDH, CD44, PTK2B, PSAP, FASN, RBM47, COL1A1, SPARC, MYC, CSN3, CSN2
2	Mammary gland development	4	1.8×10^{-4}	XDH, FASN, CSN3, CSN2
3	Single-organism developmental process	13	2.3×10^{-4}	XDH, PSAP, SPARC, CD44, PTK2B, PACSIN2, FASN, RBM47, COL1A1, GPAM, MYC, CSN3, CSN2
4	Lactation	3	2.7×10^{-4}	XDH, CSN3, CSN2
5	Gland development	5	3.6×10^{-4}	XDH, PSAP, FASN, CSN3, CSN2
6	Response to chemical	10	6.3×10^{-4}	XDH, CD44, PTK2B, PSAP, FASN, SLC41A1, COL1A1, SPARC, MYC, GPAM
7	Negative regulation of cell death	6	6.4×10^{-4}	CD44, PTK2B, PSAP, BAG3, MYC, GPAM
8	Regulation of cellular response to stress	5	1.0×10^{-3}	XDH, CD44, PTK2B, PSAP, MYC
9	Secretion by tissue	3	1.3×10^{-3}	XDH, CSN3, CSN2
10	Anatomical structure development	12	1.3×10^{-3}	XDH, CD44, PTK2B, PSAP, PACSIN2, FASN, RBM47, COL1A1, SPARC, MYC, CSN3, CSN2
11	Body fluid secretion	3	1.4×10^{-3}	XDH, CSN3, CSN2

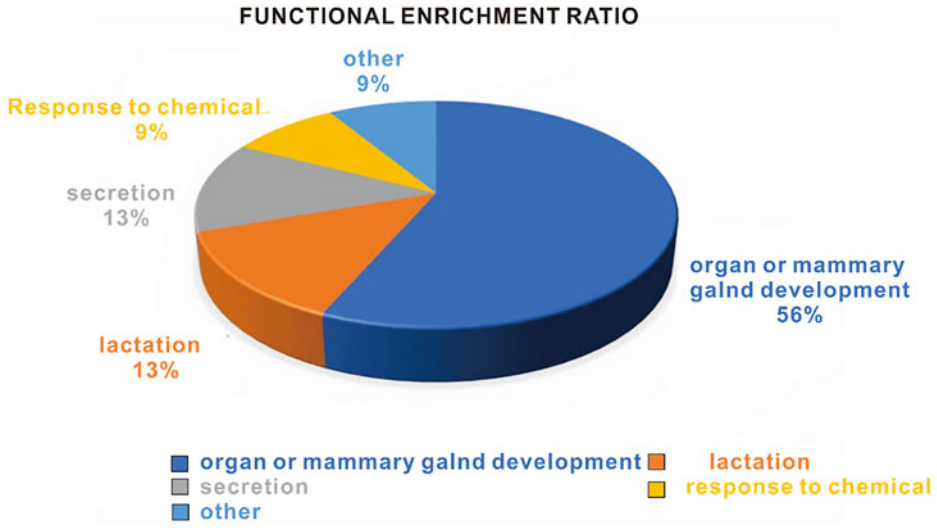

Fig. 4. Functional enrichment ratio of the genes potentially associated with in yak mammary tissue during the early lactation stage.

growth, migration, morphology, proliferation, differentiation, and biosynthetic activities (Noel and Foidart, 1998). Dhorne-Pollet *et al.* (2012) found that the expression of *COL1A1* remains low in low-milk flow ewes. This observation suggested that milking ability in ewes potentially depends on the capacity of the teat sphincter to relax during mechanical milking. In our study, the high expression of *COL1A1* in yak mammary gland during ELS implied the enhancement of mammary gland activities that prepare for lactation (Fig. 2b).

DNA sequence analysis for the identification of gene annotation

After multiple alignments, 25 unique ESTs were identified from the 56 clones (Table 1). Six genes among the 25 ESTs related to milk fat metabolism were as follows: fatty acid synthase (*FASN*), butyrophilin subfamily 1 member A1 (*BTN1A1*), glycerol phosphate acyltransferase (*GPAM*), fatty acid binding protein 3 (*FABP3*), xanthine oxidoreductase (*XDH*) and glycosylation-dependent cell adhesion molecule 1 (*GLYCAM1*). Three genes related to casein synthesis from the 25 ESTs were as follows: casein alpha s1 (*CSN1S1*), casein beta (*CSN2*) and casein kappa (*CSN3*). Both the identity of ribosomal protein S8 (*RPS8*) and *COL1A1* were up to 100%. Protein kinase C and casein kinase substrate in neurons 2 (*PACSIN2*) and v-myc myelocytomatosis viral oncogene homolog (*MYC*) have a lower identity than others. The annotation of all genes was listed in Table 1.

mRNA expression pattern analysis of selected gene

9 genes were confirmed increased at ELS compared with MLS (Fig. 3). *LAO1*, *COL1A1*, protein tyrosine kinase 2 beta (*PTK2B*), creb regulated transcription coactivator 1 (*CRTC1*) and *RPS8* were increased by 18.77, 3.87, 2.86, 2.84, and 1.92-fold, respectively. *LAO1* and *COL1A1* were the most significantly increased genes measured, which was consistent with the

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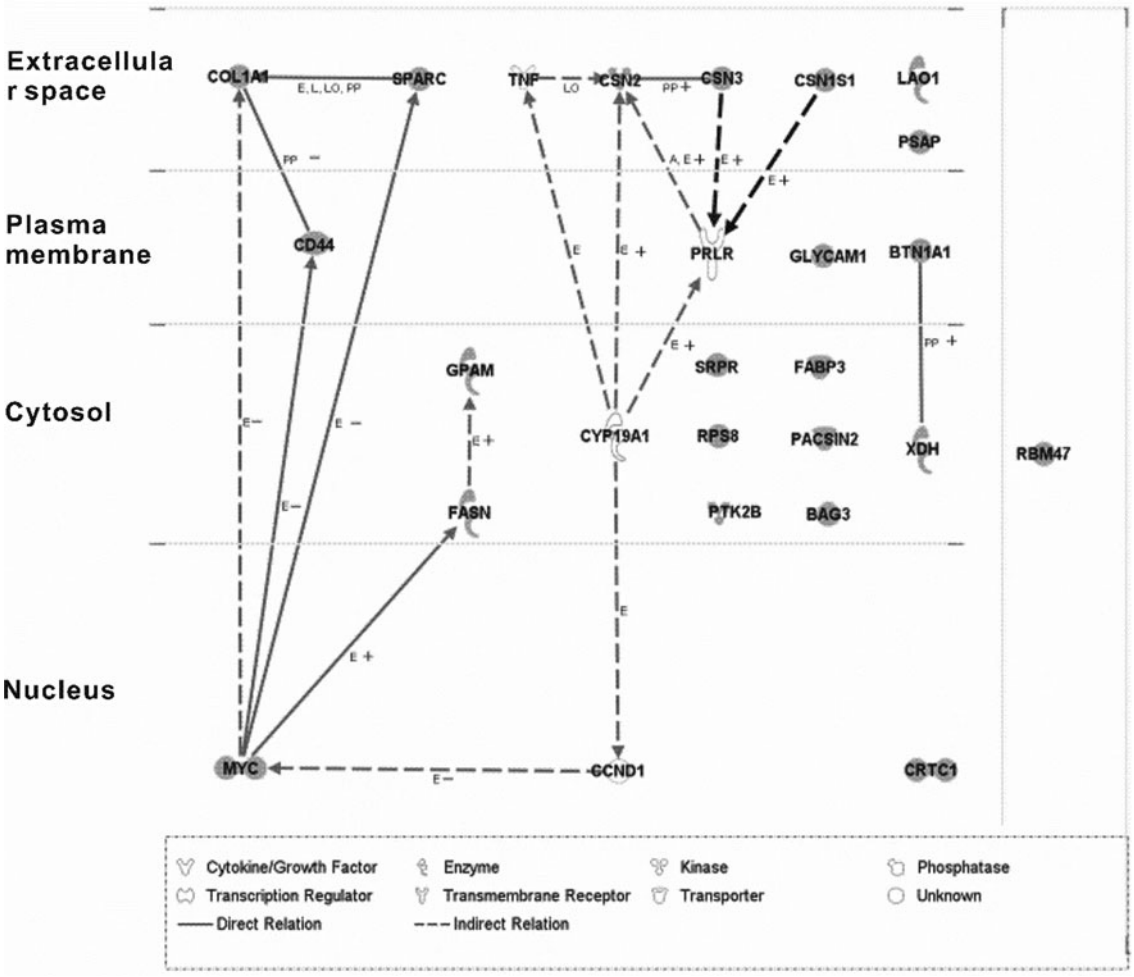

Fig. 5. Gene network pathways of the genes potentially associated with in yak mammary tissue during the early lactation stage. *Notes*: Interactions and cellular location of genes differentially-expressed at ELS relative to MLS. Networks were developed with Ingenuity Pathway Analysis (Ingenuity Systems, Inc.). Solid lines denote direct interactions and dotted lines denote indirect interactions between genes. Edge labels denote Activation/deactivation (A), effects on gene expression (E), protein–protein interactions (PP), and effect on LO. Arrows denote the direction of the effect. Symbols denote positive activation (–) or inhibition (+). To clarify the relationships among casein genes we included known relationships with PRLR and CYP19A1.

result from SSH. Prosaposin (*PSAP*) was also expressed consistently at both ELS and MLS (online Supplementary File Table S2). In contrast, the other 11 genes decreased at ELS compared with MLS. *CSN1S1*, *GLYCAM1*, *CSN3, FABP3*, and *CSN2* decreased by 3.2, 2.3, 1.9, 1.9, and 1.6-fold, respectively (online Supplementary File Table S2).

mRNA expression of milk fat and casein related genes during the lactation cycle

In general (and as expected), the expression of genes related to milk fat and casein synthesis increased during −15 to 120 d (Fig. 2e). Then, these genes sharply decreased after 120 d. The six genes related to milk fat, i.e., *BTN1A1*, *GPAM*, *FABP3*, *FASN*, *GLYCAM1*, and *XDH*, increased to the highest expression during the lactation. Their expression had a larger increase during −15 to 1 d and keenly decreased after 120 d (Fig. 2c, d).

FASN and *GPAM* are two enzymes encoding genes playing a central role in *de novo* lipogenesis and esterification. Both are putative candidate genes for quantitative trait loci (QTL) affecting milk production (Roy *et al*., 2005). *FABP3* in bovine mammary tissue is central for intracellular fatty acid trafficking and is up-regulated during the transition from the non-lactating period, and the maximum expression level appeared at 60 d post-partum (Bionaz and Loor, 2008). In our study, the expression of *FABP3* reached the peak at 15 d in yak mammary tissue. *BTN1A1* is an acidic glycoprotein that expresses on the apical surface of secretory cells in lactating mammary tissue, which functions in the formation of milk fat droplets when milk secretion is activated (Jack and Mather, 1990). *XDH* and *BTN1A1* interact with each other at the protein level and are essential for lipid droplet formation and secretion (Jack and Mather, 1990). In our study, the highest expression of *XDH* appeared at 30 and 120 d in yak mammary tissue. However, in Holstein dairy cows, *XDH* reached the peak at 60 d (Bionaz and Loor, 2008). In summary, the peak of *FABP3* and *XDH* in yak mammary tissue were reached earlier than that in Holstein dairy cows. This may be related to the length of gestation and lactation periods in yak. The length of yak lactation is shorter than the cows (Wiener *et al*., 2006).

Casein genes, i.e., *CSN1S1*, *CSN2*, and *CSN3*, sharply increased during −15 to 30 d in the yak (Fig. 2e). Casein comprises the major protein fraction of cow milk (approximately 80%) and is

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associated with the casein micelle (Sazanov *et al.*, 2006). Casein synthesis in lactating mammary gland is regulated by different pathways and involves a complex set of lactogenic hormones, such as PRL and GH (Travers *et al.*, 1996). In addition, IPA analysis revealed that *PRLR* as well as *CYP19A1* play roles in the up-regulation of casein gene expression (Akers, 2006) (Fig. 5).

Functional enrichment and gene network analysis of selected genes

Top enriched Gene Ontology (GO) analysis term and ratio are shown in Table 2 and Fig. 4 respectively with three or more genes that were significantly enriched ($P < 0.01$); these genes were primarily involved in organ or mammary gland development (56%), lactation (13%), secretion (13%) and response to chemical stimuli (9%). The result of network analysis of the 21 differentially-expressed genes in our experiment are depicted in Fig. 5. We observed evidence of potential co-regulation among the investigated genes, perhaps stemming from *MYC*. In previous studies, the expression of *MYC* decreases between the non-lactating period and the onset of lactation in humans (Grolli *et al.*, 1997) and bovine mammary tissue (Bionaz and Loor, 2007). Strange *et al.* (1992) reported that *MYC* was expressed in pregnant mouse mammary gland and then decreased to undetectable levels in lactating mammary gland, which is consistent with our results. *MYC* were found directly or indirectly interacting with *COL1A1*, *CD44*, and *SPARC* in the present study using the IPA analysis (Fig. 5). Modulation of cell surface proteins (*CD44*) has been implicated in the progression of mammary development (Hebbard *et al.*, 2000). *CD44* is down-regulated as non-ruminant mammary epithelial cells differentiate into milk-producing alveolar cells (Hebbard *et al.*, 2000). Furthermore, *CD44* takes part in the remodeling of the duct epithelium after lactation. In our study, the expression of *CD44* decreased during −15 to 1 d. It seems that *CD44* is down-regulated when mammary epithelial cells formed alveolar structures (Fig. 2).

Hasselaar and Sage (1992) showed that the function of *SPARC* including the regulation of cell adhesion/proliferation, cell morphology, cell cycle and synthesis/assembly of ECM. Gene network analysis revealed that *MYC* directly regulates the expression of *CD44*, *SPARC* and indirectly regulates the expression of *COL1A1*. To be specific, expression of *CD44*, *SPARC*, and *COL1A1* was repressed by *MYC* in rat c-*myc*-null cells (O'Connell *et al.*, 2003). Up-regulation of *CD44* was associated with the down-regulation of *COL1A1* in a sub-line of mouse NIH3T3 fibroblast (Rompaey *et al.*, 1999). Of course, the IPA analysis software is not built for cattle and yak, so we can only identify potential interactions.

In conclusion, we compared ELS and MLS in yak mammary gland and identified that the expression level of *LAO1* and *COL1A1* were significantly increased. Besides, during the lactation, the highest expression of *FABP3* and *XDH* in yak mammary tissue appears earlier than those in dairy cows. These results will be helpful for further research related to yak mammary gland and its milk production.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029919001006.

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