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Research article

Expression pattern and network visualization of genes involved in milk persistency in bovine mammary tissue

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Abstract

Importance of the work: While several studies investigated the transcriptome of bovine mammary tissue; the cellular, biological processes and metabolic pathways of differentially expressed genes involved in lactation period in bovine have not been completely considered.

Objectives: The objective was to identify the differentially expressed genes (DEGs) during the lactation period and the important genes involved in the effective metabolic pathways and biological processes involved in this period.

Materials & Methods: The expression profile developed from the Gene Expression Omnibus database of the NCBI consisted of mammary tissue samples of lactating cows from +1 d (n = 8), +15 d (n = 8), +30 d (n = 8), +60 d (n = 6), +120 d (n = 6), +240 d (n = 5) and 300 d (n = 5). DEGs were determined using the *limma* package. The protein-protein interaction (PPI) network of DEGs was drawn using Cytoscape and significant gene clusters were identified using the MCODE application. The metabolic pathways and biological processes of the DEGs were analyzed using ClueGo.

Results: In total, 344 DEGs were identified during lactation and eight significant clusters were recognized. CTNNB1, TNF, CDH1 and SPP1 had the highest degrees of connectivity in the PPI network. DEGs were enriched in metabolic pathways such as the adherens junction and TGF-beta signaling pathway.

Main finding: Overall, due to the important role of CTNNB1, TNF and CDH1 in the adherens junction and TGF-beta signaling pathway, overexpression of these genes may affect these pathways that are involved in lactation. Some significant DEGs, such as SPP1, could affect lactation persistency However, more experiments are needed to confirm these results.

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Introduction

The most valuable source of dairy products for individuals is contributed by dairy cattle and it depends on lactation performance (Ferreira et al., 2013). Extended lactations have a positive effect on herd productivity, animal health and fertility (Bissonnette, 2018).Lactation persistency (LP) is considered as part of a strategy to minimize stressful peak yields associated with health issues while maintaining a high level of production after peak yield, as LP not only represents the cow's ability to sustain milk secretion activity after the lactation peak but is also a function of the post calving development of the mammary gland (Bissonnette, 2018). The metabolic biological pathways during lactation are the most significant processes for identifying milk sustainability and milk production and they rely on mammary gland (MG) development (Gao et al., 2013). Information on gene regulation in the bovine MG can enhance methods that increase milk yield and lactation persistency. This gland undergoes involution cycles and regular proliferation. In addition, mammary epithelial cells (MECs) secrete and synthesize milk (Ryman et al., 2015). During lactation, molecular events occur in mammary epithelium cells and knowledge of these events would contribute to the development of novel technologies in the breeding and management of dairy cattle (Yang et al., 2016). It has been shown that MG metabolomics is an efficient method to identify the regulation of lactation, initiation and maintenance (Li et al., 2012; Rawson et al., 2012) and that it can provide direct links with the milking phenotypes. Therefore, exploring the metabolites and pathway in the MG during lactation would improve insight into the lactation mechanism. Additionally, research of the metabolism in bovine mammary tissue is complicated due to the presence of various multiple cells within the gland, including immune cells, fibroblasts, adipocytes, and blood vessels (Yang et al., 2016).

Gene expression profiles provide new opportunities to clarify the mechanisms of complex traits in farm animals (Mohammadi et al., 2018). Numerous studies related to gene expression in the bovine mammary gland have involved performing mammary biopsies, which are costly and invasive and disorganize the normal lactation process (Wickramasinghe et al., 2012; Cui et al., 2014; Bhat et al., 2019). Enhancement of high-throughput methods, including next-generation sequencing and microarrays, has provided the possibility of discovering gene expression patterns in the MG during involution, milk synthesis and the pregnancy period. Many analyses of transcriptome data have been conducted to recognize differentially expressed genes. For example, an experiment was carried out on the gene expression profile at Holstein cows on 3 d of lactation—15 d (transition), 90 d (peak) and 250 d (late); the gene expression analysis showed that a large number of genes were significantly expressed on all 3 d (Wickramasinghe et al., 2012). Few studies have been conducted to identify genes involved in milk production. For example, analysis of RNA-seq data was used to characterize the DEGs related to milk yield in high yielding and low yielding cattle. Thirty-one genes were notably expressed, illustrating that some DEGs would be the most fundamental candidate genes for milk yield (Cui et al., 2014; Bhat et al., 2019). In addition, studies have demonstrated that the lactation period plays a vital role in milk yield (Yoon et al., 2004; Bauman et al., 2006).

Understanding the dairy cattle metabolism has been significantly progressed, particularly in the biochemical functions and reactions of nutrient requirements (Drackley et al., 2006). However, because of a deficiency of in-depth comprehension of the complicated metabolic mechanisms involved during the lactation period, these mechanisms are still considered a "black box". The increasing application of omic technologies has led to better understanding of this "black box" (Loor et al., 2011), as these technologies evaluate the molecular and physiological alternation of dairy cows during lactation period (Gao et al., 2013). The aim of the current study was to identify candidate genes involved in metabolic pathways.

Materials and Methods

Genepix microarray data

The gene expression profile of GSE19055 was received from the Gene Expression Omnibus (GEO) database of the NCBI (National Center of Biotechnology Information). Eight multiparous Holstein dairy cows were used from the University of Illinois Dairy Cattle Research Unit. These data were related to mammary gland percutaneous biopsies performed at 1 d (n = 8), 15 d (n = 8), 30 d (n = 8), 60 d (n = 6), 120 d (n = 6), 240 d (n = 5) and 300 d (n = 5) relative to the lactation cycle and these data were deposited by Bionaz et al 2012).

Identification of differentially expressed genes

DEGs were recognized using the *limma* package (linear models for microarray data, Ritchie et al., 2015) and the microarray gene expression data were analyzed using this

package. When more than two groups of samples were defined, a moderated F-statistic was used that combined the t statistics for all the pairwise comparisons into an overall test of significance for each gene. The Benjamini & Hochberg false discovery rate was used to adjust p values. Significant DEGs with the threshold adjusted $p < 0.01$ were identified. Moreover, a Venn diagram was drawn for DEGs using the Venn package in the R program (Dusa, 2021).

Construction of protein-protein interaction network

Protein-protein interaction (PPI) networks play a significant role in interpreting biological processes (*Bakhshalizadeh* et al., 2021). Therefore, composing PPI networks provides new insight into the functions of proteins. In addition, interaction networks can be relevant from the point of view of systems biology; they may be able to reveal both the temporal and spatial aspects of the formation of functional cellular networks (Ge et al., 2003). The StringApp (version 1.4.0) and MCODE (version 1.5.1) applications in Cytoscape were used for visualization of the PPI network and gene cluster analysis, respectively (Doncheva et al., 2019; Shannon et al., 2003). After identifying significant DEGs, the official gene symbols of the DEGs were used in StingApp and MCODE. The cut-off parameters in MCODE were set as: k-core = 2; node score ≥ 0.2; degree ≥2 and maximum depth = 100. MCODE visualizes large networks by extracting the dense regions around a protein of interest (Bader and Hogue, 2003).

Gene ontology enrichment analysis

ClueGO (a Cytoscape plug-in) was used to recognize the main cellular compound (CC), molecular function (MF) and biological process (BP). It develops a biological interpretation of a gene list and can visualize the functionally, Go anthology and pathway group in terms of networks and charts (Bindea et al., 2009). A *Bos taurus* database was used to identify CC, MF and BP in the current study. Significance of gene ontology with a threshold value of $p \leq 0.05$ were identified.

Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis

The main metabolic Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was developed using ClueGo and pathways associated with the DEGs were evaluated using these applications. The network stringency was set to medium and the criteria value $p \leq 0.05$ was used to identify KEGG pathways. Significant DEGs were used to identify KEGG pathways.

Results

Identification of differentially expressed genes

The microarray dataset GSE19055 from the GEO database was acquired to determine the DEGs at +1 d, +15 d, +30 d, +60 d, +120 d, +240 d and +300 d. All days were compared together and in total, 344 DEGs were recognized. The complete gene list is provided in supplementary file 1. The Venn diagram indicated that the highest DEGs were related to +1 d of all the days investigated (Fig. 1).

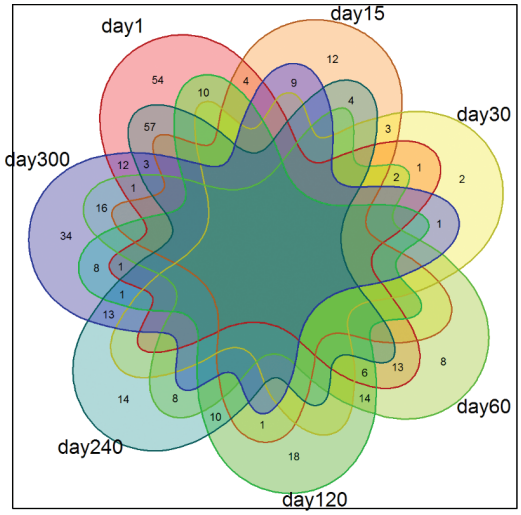

Fig. 1 Venn diagram illustrating number of differentially expressed genes in different stages in lactation period and numbers shared among these days

Construction of the protein-protein interaction network

The PPI network of these DEGs were constructed using StringApp (Fig. 2). Ultimately, there were 210 nodes and 550 edges in this network. Based on the NetworkAnalyser in Cytoscape the top 10 hub DEGs with the highest connectivity degree were screened (Table 1). These DEGs were: CTNNB1, TNF, CDH1, SPP1, CREB1, CDC5L, FYN, HNF4A, VCAM1 and B2M. These findings demonstrated that CTNNB1, TNF, CDH1 and SPP1 had the highest connectivity with the other genes in the PPI network. Significant modules were selected using MCODE; in total, eight main clusters were distinguished (Table 2).

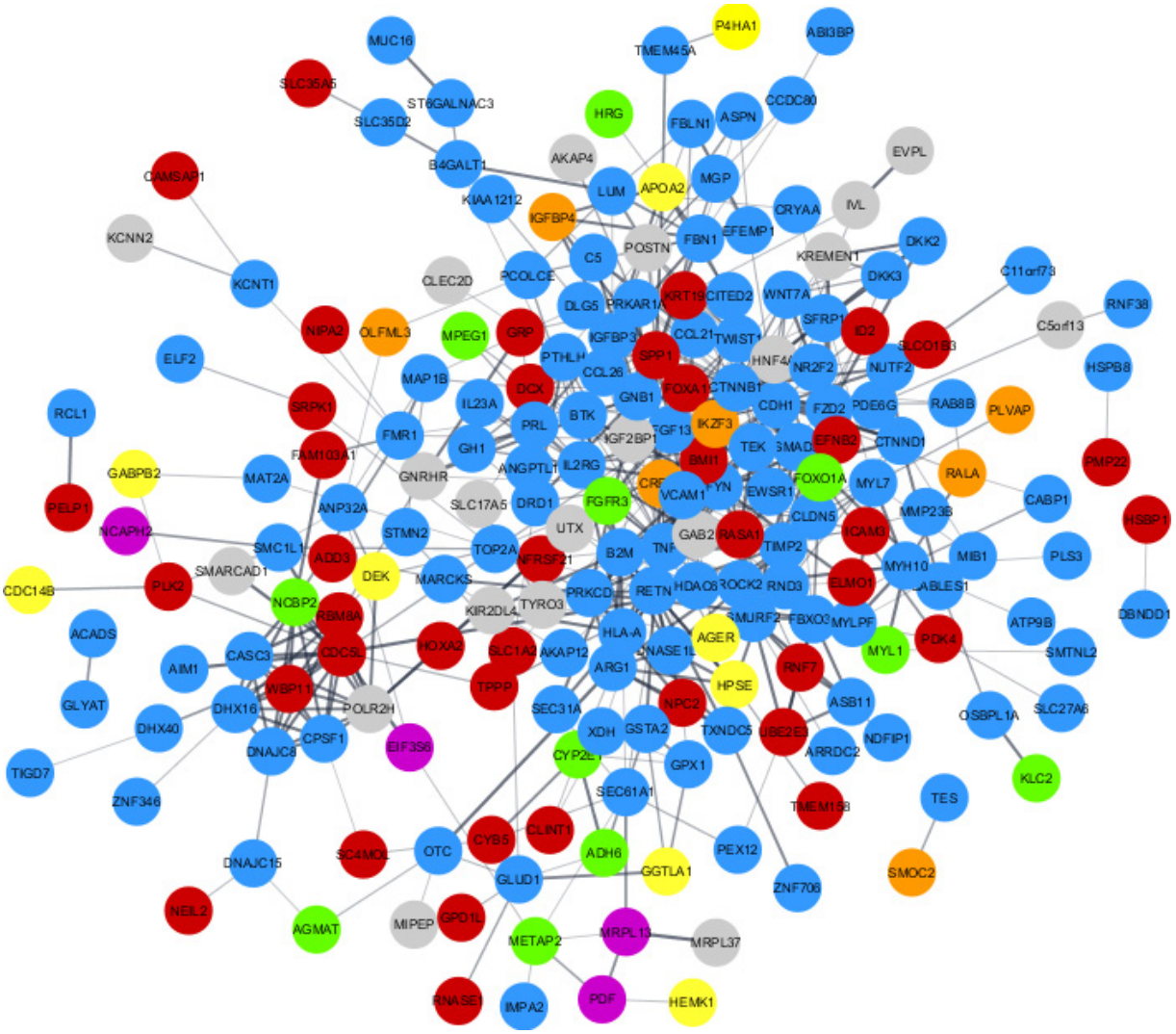

Fig. 2 Protein-protein interaction network among shared differentially expressed genes (DEGs) in lactation cycle, where each color represents DEGs related to different stages, with red, green, purple, yellow, orange and gray nodes indicating significant DEGs at +1 d, +15 d, +60 d, +120 d, +240 d and +300 d, respectively, while blue nodes denote DEGs overlapping between days

Table 1 Top-10 hub genes with highest degree in protein-protein interaction network

Gene	Degree of interaction	Closeness	Betweenness
CTNNB1	37	0.41806723	0.21654385
TNF	36	0.42340426	0.25561401
CDH1	35	0.41372141	0.14273458
SPP1	20	0.38269231	0.05218506
CREB1	19	0.38269231	0.07187905
CDC5L	17	0.30568356	0.11075951
FYN	17	0.37126866	0.04788509
HNF4A	17	0.33671743	0.00854904
VCAM1	16	0.36988848	0.02840763
B2M	15	0.33445378	0.03098863

Gene ontology enrichment analysis

DEGs enriched in BP were individually associated with regulation of cell adhesion, negative regulation of cell proliferation and tube development. In addition, DEGs enriched in CC terms were mainly associated with the proteinaceous extracellular matrix. Furthermore, DEGs enriched in MF terms were mainly associated with glycosaminoglycan binding and heparin binding (Table 3).

Table 2 Eight clusters from protein-protein interaction network

Cluster number	Score	Nodes	Edges	Differentially expressed genes
1	9	9	36	CDC5L, POLR2H, RBM8A, NCBP2, DNAJC8, DHX16, CPSF1, WBP11, CASC3
2	5.6	6	14	FZD2, SFRP1, WNT7A, DKK3, DKK2, KREMEN1
3	4.5	17	36	CDH1, CREB1, VCAM1, SMAD3, FOXO1A, TWIST1, FBXO32, SMURF2, FBN1, KRT19, BMI1, CLDN5, APOA2, RNF7, UBE2E3, IGFBP4, ASB11
4	3.6	6	9	PRKCD, ARG1, HPSE, DNASE1L1, TXNDC5, NPC2
5	3	5	6	B2M, TIMP2, IL2RG, RETN, BTK
6	3	3	3	METAP2, MRPL13, PDF
7	3	3	3	CYP2E1, GPX1, GSTA2
8	2.571	8	9	CTNNB1, FGF13, MYH10, ROCK2, RASA1, MYL7, EFNB2, NUTF2

Table 3 Gene ontology enrichment analysis of differentially expressed genes

A: Biological process			
Term	Description	Associated differentially expressed genes	*p*-value
GO:0030155	Regulation of cell adhesion	[AGER, ARG1, BMI1, CCDC80, CCL21, CDH1, CITED2, CTNNB1, DLG5, EFNB2, FBLN1, HRG, IL23A, NDFIP1, PRKAR1A, PRKCD, RASA1, SFRP1, SMOC2, TEK, TNF, TNFRSF21]	0.00
GO:0008285	Negative regulation of cell proliferation	[ARG1, B4GALT1, BTK, CTNNB1, DLG5, EFNB2, FBLN1, HNF4A, HRG, ID2, IGFBP3, NDFIP1, NR2F2, PRKAR1A, SFRP1, SMAD3, TES, TNF, TNFRSF21, XDH]	0.00
GO:0035295	Tube development	[B4GALT1, BMI1, CITED2, CREB1, CTNNB1, DLG5, EFNB2, FOXA1, FZD2, GRP, HIKESHI, ID2, KDM6A, MIB1, RALA, SFRP1, SMAD3, TNF, TWIST1, WNT7A]	0.00
GO:0010942	Positive regulation of cell death	[B4GALT1, CTNNB1, EFNB2, FOXA1, FOXO1, FYN, HDAC6, HRG, IGFBP3, PRKCD, SFRP1, SMAD3, TNF, TOP2A, XDH, ZNF346]	0.01
GO:0050865	Regulation of cell activation	[AGER, ARG1, BMI1, BTK, CTNNB1, DLG5, EFNB2, HRG, IKZF3, IL23A, NDFIP1, PRKAR1A, PRKCD, SFRP1, TNFRSF21, TYRO3]	0.00
GO:0035239	Tube morphogenesis	[B4GALT1, CITED2, CTNNB1, DLG5, EFNB2, FOXA1, FZD2, ID2, KDM6A, MIB1, RALA, SFRP1, SMAD3, TNF, TWIST1]	0.00
GO:0048568	Embryonic organ development	[BMI1, CITED2, CRYAA, CTNNB1, EFEMP1, FBN1, FZD2, ID2, KDM6A, KRT19, MIB1, NR2F2, SMAD3, TNF, TWIST1]	0.01
GO:0019216	Regulation of lipid metabolic process	[APOA2, CCL21, CREB1, DNAJC15, FGFR3, GH1, HNF4A, ID2, PDK4, PRKCD, PRL, RETN, TEK, TNF, TWIST1]	0.00
GO:0022407	Regulation of cell-cell adhesion	[AGER, ARG1, BMI1, CCL21, CDH1, CITED2, CTNNB1, DLG5, EFNB2, IL23A, NDFIP1, PRKAR1A, PRKCD, TNF, TNFRSF21]	0.00
GO:0060562	Epithelial tube morphogenesis	[B4GALT1, CITED2, CTNNB1, DLG5, EFNB2, FOXA1, FZD2, KDM6A, MIB1, RALA, SFRP1, SMAD3, TNF, TWIST1]	0.00
GO:0002683	Negative regulation of immune system process	[APOA2, ARG1, BTK, C5, CCL21, CTNNB1, DLG5, FBN1, NDFIP1, PRKAR1A, SFRP1, TNF, TNFRSF21, TYRO3]	0.01

Table 3 Coutinued

A: Biological process			
Term	Description	Associated differentially expressed genes	*p*-value
GO:0051249	Regulation of lymphocyte activation	[AGER, ARG1, BMI1, BTK, CTNNB1, DLG5, EFNB2, IKZF3, IL23A, NDFIP1, PRKAR1A, SFRP1, TNFRSF21, TYRO3]	0.01
GO:0032943	Mononuclear cell proliferation	[AGER, ARG1, BMI1, BTK, CTNNB1, DLG5, FYN, IKZF3, IL23A, NDFIP1, PRKAR1A, PRKCD, TNFRSF21]	0.00
GO:0046651	Lymphocyte proliferation	[AGER, ARG1, BMI1, BTK, CTNNB1, DLG5, FYN, IKZF3, IL23A, NDFIP1, PRKAR1A, PRKCD, TNFRSF21]	0.00
GO:1903037	Regulation of leukocyte cell-cell adhesion	[AGER, ARG1, BMI1, CCL21, CTNNB1, DLG5, EFNB2, IL23A, NDFIP1, PRKAR1A, TNF, TNFRSF21]	0.01
GO:0045834	Positive regulation of lipid metabolic process	[APOA2, CCL21, CREB1, FGFR3, GH1, PRKCD, PRL, RETN, TEK, TNF, TWIST1]	0.00
GO:0007162	Negative regulation of cell adhesion	[ARG1, CCL21, CDH1, DLG5, FBLN1, HRG, NDFIP1, PRKAR1A, PRKCD, RASA1, TNFRSF21]	0.00
GO:0070663	Regulation of leukocyte proliferation	[AGER, ARG1, BMI1, BTK, CTNNB1, DLG5, IKZF3, IL23A, NDFIP1, PRKAR1A, TNFRSF21]	0.00
GO:0032944	Regulation of mononuclear cell proliferation	[AGER, ARG1, BMI1, BTK, CTNNB1, DLG5, IKZF3, IL23A, NDFIP1, PRKAR1A, TNFRSF21]	0.00
GO:0050670	Regulation of lymphocyte proliferation	[AGER, ARG1, BMI1, BTK, CTNNB1, DLG5, IKZF3, IL23A, NDFIP1, PRKAR1A, TNFRSF21]	0.00
GO:0042098	T cell proliferation	[AGER, ARG1, BMI1, CTNNB1, DLG5, FYN, IL23A, NDFIP1, PRKAR1A, TNFRSF21]	0.00
GO:0050866	Negative regulation of cell activation	[ARG1, BTK, DLG5, NDFIP1, PRKAR1A, PRKCD, SFRP1, TNFRSF21, TYRO3]	0.01
GO:0042129	Regulation of T cell proliferation	[AGER, ARG1, BMI1, CTNNB1, DLG5, IL23A, NDFIP1, PRKAR1A, TNFRSF21]	0.00
GO:0051250	Negative regulation of lymphocyte activation	[ARG1, BTK, DLG5, NDFIP1, PRKAR1A, SFRP1, TNFRSF21, TYRO3]	0.01
GO:0034103	Regulation of tissue remodeling	[AGER, B4GALT1, HRG, PDK4, SFRP1, SPP1]	0.01
GO:0045670	Regulation of osteoclast differentiation	[CREB1, CTNNB1, FBN1, IL23A, SFRP1, TNF]	0.01
GO:0050994	Regulation of lipid catabolic process	[APOA2, GH1, PRKCD, RETN, TNF, TWIST1]	0.00
GO:0032945	Negative regulation of mononuclear cell proliferation	[ARG1, BTK, DLG5, NDFIP1, PRKAR1A, TNFRSF21]	0.01
GO:0050672	Negative regulation of lymphocyte proliferation	[ARG1, BTK, DLG5, NDFIP1, PRKAR1A, TNFRSF21]	0.01
GO:0050996	Positive regulation of lipid catabolic process	[APOA2, GH1, PRKCD, RETN, TWIST1]	0.00
GO:1903727	Positive regulation of phospholipid metabolic process	[CCL21, FGFR3, GH1, PRKCD, TEK]	0.01
GO:0007595	Lactation	[CREB1, GH1, PRL, TNF, XDH]	0.00

Table 3 Coutinued

B: Cellular component			
Term	Description	Associated DEGs	*p*-value
GO:0005578	Proteinaceous extracellular matrix	[ASPN, CCDC80, CCL21, FBLN1, FBN1, HPSE, LUM, POSTN, SFRP1, SMOC2, TIMP2, WNT7A]	0.00
GO:0098589	Membrane region	[BTK, CDH1, CTNNB1, FYN, HDAC6, HPSE, PLVAP, PRKAR1A, TEK, TNF]	0.01
GO:0098857	Membrane microdomain	[BTK, CDH1, CTNNB1, FYN, HDAC6, HPSE, PLVAP, PRKAR1A, TEK, TNF]	0.00
GO:0045121	Membrane raft	[BTK, CDH1, CTNNB1, FYN, HDAC6, HPSE, PLVAP, PRKAR1A, TEK, TNF]	0.00
GO:0044853	Plasma membrane raft	[CDH1, CTNNB1, HDAC6, PLVAP, PRKAR1A]	0.04
C: Molecular function			
Term	Description	Associated DEGs	*p*-value
GO:0005539	Glycosaminoglycan binding	[AGER, CCDC80, FBN1, HRG, PCOLCE, POSTN, SFRP1, SMOC2]	0.01
GO:0008201	Heparin binding	[AGER, CCDC80, FBN1, HRG, PCOLCE, POSTN, SFRP1, SMOC2]	0.00
GO:0004713	Protein tyrosine kinase activity	[BTK, EFEMP1, FGFR3, FYN, TEK, TYRO3]	0.03
GO:0005179	Hormone activity	[FBN1, GH1, GRP, PRL, PTHLH, RETN]	0.03
GO:0044325	Ion channel binding	[CTNNB1, FGF13, FMR1, FYN, GPD1L, ID2]	0.01
GO:0033613	Activating transcription factor binding	[CITED2, CREB1, CTNNB1, HNF4A, SMAD3]	0.01
GO:0001102	RNA polymerase II activating transcription factor binding	[CITED2, CREB1, CTNNB1, HNF4A, SMAD3]	0.00
GO:0005518	Collagen binding	[ASPN, LUM, PCOLCE, SMAD3]	0.04
GO:0001968	Fibronectin binding	[CCDC80, FBLN1, IGFBP3]	0.01

Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis

KEGG pathway enrichment analysis showed that DEGs were notably involved in 15 pathways such as the adherens junction and the TGF-beta signaling pathway (Fig. 3).

Discussion

The purpose of this study was to identify the DEGs in the lactation period in Holstein cows and to identify the relevant pathway to understand the underlying molecular mechanisms. Bionaz et al. (2012) explored the cow mammary transcriptome at -30 d, -15 d, +1 d, +15 d, +30 d, +60 d, +120 d, +240 d and +300 d relative to parturition. Boussadia et al. (2002) conducted functional analysis of microarray data using the dynamic impact approach and their overall results revealed that the bovine MG is heavily dependent on a coordinated transcriptional regulation to begin and end lactation. The functional research using dynamic impact approach (DIA) underscored the significance of genes associated with immune function, angiogenesis, lipid metabolism, protein synthesis, epigenetic regulation, Golgi, transport, cell cycle/death and lactose synthesis during lactation. The current study screened 344 DEGs at +1 d, +15 d, +30 d, +60 d, +120 d, +240 d and +300 d. The results indicated that CTNNB1, TNF, CDH1 and SPP1 had the highest degrees of interaction in the protein-protein interaction network. Furthermore, these genes were reported as DEGs in a study that explored DEGs in the MG epithelial cells at different stages of lactation (+15 d, +90 d, 250 d) between Jersey and Kashmiri cattle (Bhat et al., 2019).

The current results indicated that SPP1 (osteopontin) had a high connectivity degree in the PPI network. This gene is a secreted glycophosphoprotein, which has a vital role in MG development, and it has been involved in different cancers especially breast cancer (Shevde et al., 2010). In addition, it has an extremely high expression level in the mouse lactation gland (Rittling and Novick, 1997) and it is part of the molecular mechanisms involved during involution of the MG in mice (Baik et al., 1998; Prokesch et al., 2014). Targeted inhibition of SPP1 expression in a mouse caused lactation deficiency

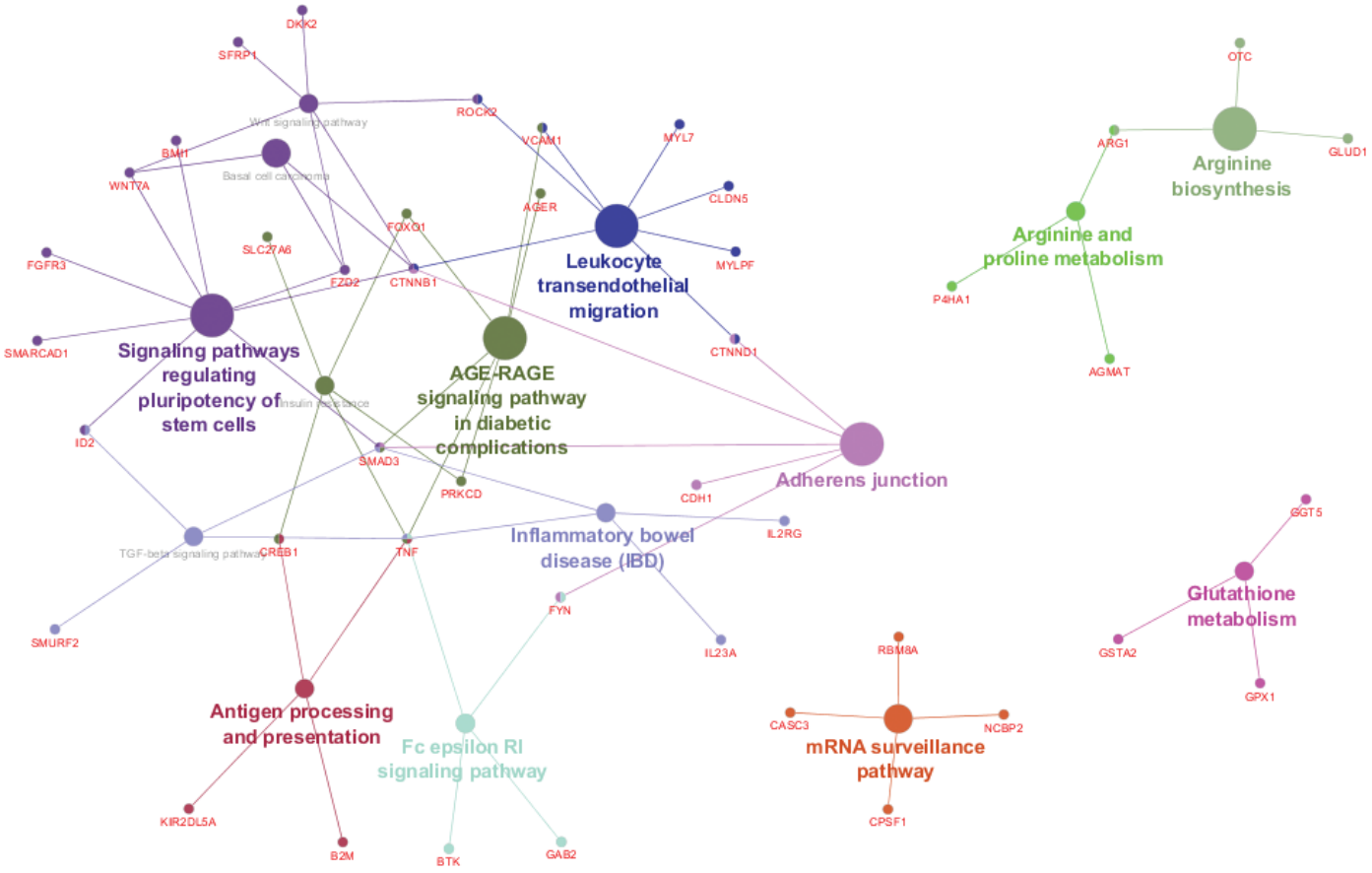

Fig. 3 The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for differentially expressed genomes (DEGs) visualized using Cytoscape with the ClueGo application, where KEGG pathways are represented as nodes based on their kappa score level and large nodes show the enriched pathway, whereas small nodes indicate DEGs involved in each pathway

(Nemir et al., 2000). The expression of this gene has been persistent during lactation in humans (Nagatomo et al., 2004) and SPP1 has been used as a marker to recognize differentiated secretory mammary epithelial cells (Brown et al., 1992). Furthermore, SPP1 increasingly is expressed in the cattle MG during bacterial infection to enhance cell-mediated immunity (Denhardt et al., 2001; Konno et al., 2006; Alain et al., 2009). Genetic variations of SSP1 impact on the amount of its secretion throughout lactation in bovine species (Dudemaine et al., 2014). In addition, SPP1 could influence lactation persistency in dairy cows. Accordingly, SPP1 has been reported as a candidate gene associated with lactation persistency for dairy cattle (Bissonnette, 2018). Furthermore, 4 single nucleotide polymorphisms (SNPs) in **SPP1** were determined to be associated with the mammary health status of dairy cows (Alain et al., 2009). According to other studies (Shevde et al., 2010; Hubbard et al., 2013), it seems that SPP1 expression has a crucial role for MG development and the dysregulation of this gene based on its role in cell migration, survival and invasion and in critical steps for developing epithelial tissues embedded in a stroma; thus, it could cause major changes and it might play a role in mammary tumorigenesis.

Furthermore, the current results indicated that CDH1 (E-cadherin) had a high degree of connectivity in the PPI network for the shared DEGs. It is expressed particularly in all mammary epithelial cells (Hazan et al., 1997; Paredes et al., 2002a; Paredes et al., 2002b). E-cadherin underpins a strong connection between epithelial cells and components of the adherens junction which are localized and interact in normal mammary epithelial cells (Paredes et al., 2002a). E-cadherin has been recognized as a tumor suppressor gene in different cancers such as breast cancer (Tryndyak et al., 2010). Because E-cadherin enhances the development and adhesion of a normal epithelial phenotype and its architecture, its down-regulation in cancer cells is related to an increase in overall metastasis, epithelial-mesenchymal transition, invasiveness and motility

(Bracke et al., 1997; Tryndyak et al., 2010). Hence, the epidermal growth factor receptor interacts with E-cadherin in order to suppress receptor tyrosine kinase signaling, which regulates cell proliferation (Fedor-Chaiken et al., 2003). This protein was present at the adherens junctions in a differentiated mammary epithelial cell. According to the gene ontology and KEGG pathway enrichment analysis in the current study, this gene was enriched in several biological processes, such as adherens junction organization, regulation of cell adhesion, cell junction organization, cell-cell junction organization and the Adherens junction KEGG pathway, respectively. Furthermore, corresponding to our results, CTNBB1 (B-catenin) which had the highest degree of connectivity in the current PPI network, had common pathways with CDH1, especially the Adherens junction (AJ) KEGG pathway. This pathway provides important adhesive connections between neighboring epithelial cells. The major function of the AJ is to sustain a physical connection between cells and these junctions are implicated in differentiation, cell polarity, structure, initiation, migration, growth and the stabilization of cell-cell adhesion (Gumbiner, 1996, 2005; Larue et al., 1996).Then, their loosening is related to a lack of cell-cell contact, leading to a disturbed tissue structure (Breier et al., 2014; Schneider and Kolligs, 2015; van Roy, 2014). In epithelial cells, AJs contain two types of proteins: catenins, including β-catenin, and the transmembrane cadherins, such as E-cadherin and the cytoplasmic domain of cadherin (Gumbiner, 2005). These proteins regulate the function of AJs. In the MG, E-cadherin is widely expressed in epithelial cells from the early embryonic stage until the organs have matured. Consequently, it was expected that E-cadherin was essential for the formation of epithelial tissues. To test the consequences of E-cadherin loss in the lactating mammary gland and pregnancy, conditional gene deletion was used that indicated E -cadherin plays a vital role in the function and survival of alveolar epithelial cells (Boussadia et al., 2002). β-Catenin, which binds to cadherins, indicates an analogous localization in luminal cells and is expressed in the stroma and myoepithelial cells (Michaelson and Leder, 2001; Incassati et al., 2010). The β-catenin survival signal is necessary for normal development of the mammary lobular gland (Tepera et al., 2003). Dysregulation of AJ proteins has been associated with cancer and mammary gland developmental deficiencies (Hoevel et al., 2004; Osanai et al., 2006, 2007; Lanigan et al., 2009;). For example, inhibition or mis-localization of β-catenin result in hyperplasia and breast cancer (Michaelson and Leder, 2001; Incassati et al., 2010; López-Knowles et al., 2010;) and due to the important role of E-cadherin in growth of epithelial cells (Lanigan et al., 2007), dysregulation of this protein is linked to lactation defects (Boussadia et al., 2002), resulting in hyperplasia and dysplasia with age (Radice et al., 2003) and premature differentiation of the gland and breast cancer (Paredes et al., 2005). Furthermore, the current analysis showed that β-catenin was enriched in the Wnt signaling pathway, where it is an essential protein component (Hatsell et al., 2003). β-Catenin might bind to the transcription factor and interfere with the transcription of genes that are implicated in cell proliferation of the Wnt signaling pathway (Nelson and Nusse, 2004). The Wnt signaling pathway is implicated in many stages of differentiation and growth of the MG (Smalley and Dale, 2001; Hatsell et al., 2003; Howe and Brown, 2004), and it could be involved in the involution of mammary tissue that occurs in lactation (Lacher et al., 2003). Overall, it was concluded that dysregulation of E-cadherin and β-catenin impacts on defects in MG development and lactation.

TNF had a high nodal degree in the PPI network in the current study. Other studies have shown that this protein plays a vital role in the MG. For example, during lactation, TNF may stimulate functional differentiation, leading to extensive secretion and synthesis of milk proteins at this developmental stage. Hence, TNF inhibits the accumulation of casein protein in primary cultures of mammary epithelial cells (Ip et al., 1992; Varela and Ip, 1996; Varela et al., 1997; Lee et al., 2000). The KEGG enrichment analysis in the current study indicated that this gene was enriched in some pathways, such as TGF-beta signaling pathway. Transforming growth factor beta (TGFβ) presents in three various isoforms (TGFβ 1, 2 and 3) which belong to the growth factor family (Gilbert et al., 2016). TGFβ1 plays a significant role in the involution of the bovine MG by inducing autophagy and apoptosis in bovine mammary luminal epithelial cells (Kolek et al., 2003; Gajewska et al., 2005). In addition, TGFβ1 arrests bovine epithelial cell growth (Kolek et al., 2003) and it had a significant effect on MG functional development in mice (Flanders and Wakefield, 2009). TGFβ1 is currently known as a main regulator of MG ductal patterning; when TGFβ1 was highly expressed in mastitic MGs, fibrosis and apoptosis of epithelial cells increased (Andreotti et al., 2014). It has been shown to improve invasion and adhesion of *Staphylococcus aureus* to bovine mammary fibroblasts (Zhao et al., 2017), which makes TGFβ an important target of future research to control and understand bovine mastitis. Due to the significant role of TNF, over expression of TNF might have a negative effect on the TGF-beta signaling pathway.

In conclusion, the current study identified many DEGs at +1 d, +15 d, +30 d, +60 d, +120 d, +240 d and +300 d lactation in

the bovine mammary gland using bioinformatics analysis. The shared DEGs, such as CTNNB1, TNF, CDH1 and SPP1, had high degrees of connectivity in the PPI network. The current study results suggested that these genes could affect pathways that are critical, including the Adherens junction and TGF-beta signaling pathway. Furthermore, dysfunction of these genes may impact on the function of pathways and cause defects in mammary gland development and lactation. However, more experimental research is needed to confirm these results and to explore the complex molecular mechanism in more detail.

Conflict of Interest

The authors declare that there are no conflicts of interests.

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