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Examination of methionine stimulation of gene expression in dairy cow mammary epithelial cells using RNA-sequencing

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Research Article

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Abstract

In this research communication, a cell model with elevated β-CASEIN synthesis was established by stimulating bovine mammary epithelial cells with 0.6 mM methionine, and the genome-wide gene expression profiles of methionine-stimulated cells and untreated cells were investigated by RNA sequencing. A total of 458 differentially expressed genes (DEGs; 219 upregulated and 239 downregulated) were identified between the two groups. Gene Ontology (GO) analysis showed that the two highest-ranked GO terms in 'molecular function' category were 'binding' and 'catalytic activity', suggesting that milk protein synthesis in methionine-stimulated cells requires induction of gene expression to increase metabolic activity. Kyoto Encyclopedia of Genes and Genomes analysis revealed that within the 'environmental information processing' category, the subcategory that is most highly enriched for DEGs was 'signal transduction'. cGMP-PKG, Rap1, calcium, cAMP, PI3K-AKT, MAPK, and JAK-STAT are the pathways with the highest number of DEGs, suggesting that these signaling pathways have potential roles in mediating methionine-induced milk protein synthesis in bovine mammary epithelial cells. This study provides valuable insights into the physiological and metabolic adaptations in cells stimulated with methionine. Understanding the regulation of this transition is essential for effective intervention in the lactation process.

Methionine has been identified as one of the most limiting amino acids for milk protein synthesis (Hanigan *et al*., 2002). The addition of methionine to cultured bovine mammary epithelial cells significantly increases β-CASEIN expression (Duan *et al*., 2017). Amino acids not only serve as substrates for milk protein synthesis but also function as signaling molecules that regulate the process. Evidence from work with both bovine mammary epithelial cells and lactating cows demonstrated that amino acid availability may modulate protein translation through the mammalian target of rapamycin (mTOR) signaling pathway (Appuhamy *et al*., 2011). Although methionine is a potent stimulator of protein synthesis in the mammary gland, the relationship between methionine supplementation and gene expression related to milk protein synthesis is not well understood.

Gene expression profiles offer new opportunities to elucidate the underlying mechanisms of lactation traits in dairy cows. Several studies of the bovine mammary gland transcriptome by RNA sequencing (RNA-seq) have been reported, and a number of differentially expressed genes (DEGs) and signaling pathways that play regulatory roles during lactation have been identified (Lin *et al*., 2019). However, few studies have been done in a cell model to assess gene expression changes related to milk protein synthesis induced by individual amino acids. It is straightforward to stimulate cells *in vitro* by the addition of an individual amino acid in the medium. Although a mammary epithelial cell model of milk protein synthesis does not replicate *in vivo* lactation, its use for gene expression analysis eliminates some of the interference from other cell types. Such a model is thus essential for identifying candidate genes that contribute to milk component synthesis in bovine mammary gland.

Therefore, we established a cell model by stimulating cultured bovine mammary epithelial cells with methionine to produce higher levels of milk protein. RNA sequencing was used to examine the genome-wide gene expression profiles between mammary epithelial cells treated with methionine and untreated cells. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) tools were used to analyze functional enrichment. This study provides valuable insights into the physiological and metabolic adaptations in cells treated with methionine. Understanding the regulation of this transition is essential for effective intervention in lactation process.

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Materials and methods

All experimental procedures were approved by the Northeast Agricultural University Animal Care and Use Committee (Harbin, China). Primary mammary epithelial cells were isolated from lactating bovine mammary tissue and cultured in Dulbecco's modified Eagle's medium : Nutrient Mixture F-12 (DMEM/F12, Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (BI Biological Industries, Kibbutz Beit-Haemek, Israel), 100 U/ml penicillin, 100 μg/ml streptomycin, and lactating hormones (5 μg/ml insulin, 1 μg/ml prolactin, and 1 μg/ml hydrocortisone; Sigma-Aldrich, St Louis, MO) (Duan *et al*., 2017). CYTOKERATIN 18 expression was detected by immunofluorescence (detailed procedures are described in the online Supplementary File). To establish a cell model with a high capacity for milk protein synthesis, mammary epithelial cells were seeded in 6-well plates at a density of 2×10^5 cells/well. When cells grew to ~90% confluence, methionine was added to the medium at a concentration of 0.6 mM (Duan *et al*., 2017). After 24 h of treatment, cells were collected for the detection of β-CASEIN expression by western blot analysis (detailed procedures are described in the online Supplementary File). Cells untreated with methionine were used as the control.

For RNA-seq analysis, total RNA was isolated from three methionine-stimulated samples and three control samples using a Magen HiPure Total RNA Mini kit (Magen, Guangzhou, China). Concentrations and qualities of RNA samples were measured using NanoDrop 2000 (Thermo Scientific, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All samples used in this study had an RNA integrity number >8. An RNA-seq transcriptome library was prepared for each sample using 5 μg of total RNA using the TruSeqTM RNA sample preparation kit from Illumina (San Diego, CA). mRNA was isolated by oligo(dT) beads and then fragmented by fragmentation buffer. The SuperScript double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA) was used to synthesize cDNA with random hexamer primers (Illumina). The cDNA was subjected to end repair, phosphorylation, and 'A' base addition. After 15 cycles of PCR, libraries were size-selected for cDNA target fragments of 200–300 bp on 2% low-range ultra agarose. Then the paired-end RNA-seq libraries were quantified by TBS380 (American Instrument Exchange, Inc., Haverhill, MA) and were sequenced using the Illumina HiSeq 4000 (2×150 bp read length).

According to the fragments per kilobase of exon per million mapped reads method, the expression level of each gene was calculated. RNA-seq by Expectation-Maximization was used to quantify gene abundances, and empirical analysis of digital gene expression in R (EdgeR) was used for differential expression analysis. The DEGs between methionine-stimulated and control samples were analyzed by Goatools (https://github.com/tanghaibao/Goatools) and KEGG orthology-based annotation system (KOBAS, http://kobas.cbi.pku.edu.cn/home.do) to identify the GO terms and biological pathways in which DEGs were significantly enriched at Bonferroni-corrected P value ≤0.05 compared with the whole transcriptome background.

Six upregulated DEGs (*MCOLN2*, *JAZF1*, *TNF24*, *LIFR*, *KLHL28*, *SLC12A5*) and six downregulated DEGs (*MAP6D1*, *PPIL6*, *NOSTRIN*, *SEC14L5*, *TMC2*, *FAM71A*) were randomly selected for quantitative reverse transcription-PCR (qRT-PCR) to validate the RNA-seq results. Reverse transcription and real-time PCR were performed with the PrimeScript^{RT} Reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China) and ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The expression level of each gene was normalized to β–*ACTIN*. All primer sequences are listed in online Supplementary Table S1.

Results and discussion

We established a cell model that produces a high level of milk protein by stimulating purified bovine mammary epithelial cells with 0.6 mM methionine for 24 h. Immunofluorescence showed that the marker of mammary epithelial cells, CYTOKERATIN 18, was expressed in all cells (online Supplementary Fig. 1A). The expression of β-CASEIN was significantly higher in cells stimulated with methionine than in the control cells ($P < 0.01$; online Supplementary Fig. 1B, 1C), confirming that methionine stimulation induced mammary epithelial cells to increase production of milk protein.

We identified 458 DEGs (219 upregulated and 239 downregulated) that were significantly differentially expressed between methionine-stimulated cells and control cells based on a $|\log_2 \text{ratio}| \geq 1$ and P value ≤0.05 rule. Details of DEGs are shown in Table 1 and online Supplementary Fig. S2. We randomly selected six upregulated and six downregulated DEGs to validate the RNA-seq results by qRT-PCR. The expression levels calculated *via* RNA-seq and qRT-PCR were significantly positively correlated ($R^2 = 0.9092$, $P < 0.0001$; online Supplementary Fig. S3), confirming that our RNA-seq analysis was successful and accurate.

To investigate the functional associations of all 458 DEGs, the DEGs were analyzed with the GO database. The two highest-ranked GO terms in the 'molecular function' category were 'binding' and 'catalytic activity' (online Supplementary Fig. S4), suggesting that milk protein synthesis in methionine-stimulated cells requires induction of gene expression to increase metabolic activity.

To identify biological pathways that may have been activated in methionine-simulated cells, KEGG analysis was performed with the KOBAS database. Our data showed that DEGs were most common in the 'translation' and 'folding, sorting and degradation' subcategories of the 'genetic information processing' category (online Supplementary Fig. S5), reflecting an increased translational and post-translational modification requirement associated with the synthesis of milk proteins and enzymes in methionine-stimulated cells. KEGG analysis also revealed that DEGs were most enriched in the 'signal transduction' subcategory of the 'environmental information processing' category (online Supplementary Fig. S5). cGMP-PKG, Rap1, calcium, cAMP, PI3K-AKT, MAPK, and JAK-STAT are the pathways with the highest number of DEGs (Fig. 1).

It is known that milk protein production is a function of the number and activity of secretory epithelial cells. Activation of the mitogen-activated protein kinase (MAPK) signaling pathway is associated with the increased expression of a wide range of genes that promote cell survival and proliferation (Hicks *et al*., 2015). Our previous study showed that the viability of bovine mammary epithelial cells is significantly increased in cells treated with 0.6 mM methionine (Duan *et al*., 2017). In the current study, we found that four upregulated genes (*MAP3K13*, *AKT3*, *FGF21*, and *FGFR4*) were enriched in the MAPK signaling pathway, which is consistent with the effect of methionine on mammary epithelial cell survival and proliferation. Additionally, among these gene products, fibroblast growth factor 21 (FGF21) is a

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Table 1. Top 50 upregulated and downregulated genes between methionine-stimulated cells and control cells

Gene name	Gene ID	Gene description	$\log_2$ FC	*P* value	FDR
Upregulated					
ANKRD34A	538433	ankyrin repeat domain 34A	4.95	7.85×10^{-05}	1.58×10^{-04}
TMEM88	507172	transmembrane protein 88	4.86	1.34×10^{-04}	2.54×10^{-04}
SCUBE3	100300124	signal peptide, CUB domain and EGF like domain containing 3	4.73	2.85×10^{-04}	4.87×10^{-04}
SMOC2	615490	SPARC-related modular calcium-binding protein 2 precursor	4.60	5.47×10^{-04}	8.65×10^{-04}
BCL6B	100138731	B-cell CLL/lymphoma 6B	4.50	4.06×10^{-16}	3.93×10^{-15}
RAB3B	282030	RAB3B, member RAS oncogene family	4.31	2.07×10^{-03}	2.77×10^{-03}
TNFRSF11A	530884	TNF receptor superfamily member 11a	4.14	4.06×10^{-03}	4.94×10^{-03}
KCNQ5	613605	potassium voltage-gated channel subfamily Q member 5	4.05	9.28×10^{-05}	1.84×10^{-04}
PRKG1	282004	protein kinase, cGMP-dependent, type I	3.95	1.79×10^{-04}	3.26×10^{-04}
RAMP1	617017	receptor activity-modifying protein 1 precursor	3.95	8.05×10^{-03}	8.88×10^{-04}
CPS1	510506	carbamoyl-phosphate synthase 1	3.95	8.05×10^{-03}	8.88×10^{-03}
GRID1	100337106	glutamate ionotropic receptor delta type subunit 1	3.95	8.05×10^{-03}	8.88×10^{-03}
WDR38	787677	WD repeat domain 38	3.85	3.40×10^{-04}	5.70×10^{-04}
FGFR4	317696	fibroblast growth factor receptor 4	3.73	6.67×10^{-04}	1.03×10^{-03}
IDO1	506281	indoleamine 2,3-dioxygenase 1	3.73	6.67×10^{-04}	1.03×10^{-03}
HILS1	615867	*Bos taurus* histone linker H1 domain, spermatid-specific 1 (HILS1), mRNA	3.73	1.61×10^{-02}	1.59×10^{-02}
CASQ2	528555	calsequestrin 2	3.73	1.61×10^{-02}	1.59×10^{-02}
CXCL12	613811	C-X-C motif chemokine ligand 12	3.46	2.51×10^{-03}	3.28×10^{-03}
LOC 100848478	100848478	60S ribosomal protein L7a pseudogene	3.46	3.26×10^{-02}	2.84×10^{-02}
DUSP13	616048	dual specificity phosphatase 13	3.31	4.89×10^{-03}	5.80×10^{-03}
ACSBG1	515577	acyl-CoA synthetase bubblegum family member 1	3.14	9.53×10^{-03}	1.03×10^{-02}
EFCAB3	524320	EF-hand calcium binding domain 3	3.14	9.53×10^{-03}	1.03×10^{-02}
PLCL1	537873	phospholipase C like 1	3.14	9.53×10^{-03}	1.03×10^{-02}
NEK11	614924	NIMA related kinase 11	3.14	9.53×10^{-03}	1.03×10^{-02}
PIEZO2	522631	piezo type mechanosensitive ion channel component 2	3.14	9.53×10^{-03}	1.03×10^{-02}
AMY2B	505049	amylase, alpha 2B	3.02	1.09×10^{-06}	3.17×10^{-06}
GHR	280805	growth hormone receptor	2.95	8.73×10^{-04}	1.31×10^{-03}
DHH	525833	desert hedgehog	2.95	1.86×10^{-02}	1.79×10^{-02}
RIMS2	535674	regulating synaptic membrane exocytosis 2	2.95	1.86×10^{-02}	1.79×10^{-02}
ADAMTSL2	526780	ADAMTS-like protein 2 precursor	2.95	1.86×10^{-02}	1.79×10^{-02}
TEX14	522810	testis expressed 14, intercellular bridge forming factor	2.95	1.86×10^{-02}	1.79×10^{-02}
NCR3	513769	natural cytotoxicity triggering receptor 3	2.95	1.86×10^{-02}	1.79×10^{-02}
SYT17	532273	synaptotagmin 17	2.84	1.64×10^{-03}	2.26×10^{-03}
MCOLN2	532671	mucolipin 2	2.84	1.64×10^{-03}	2.26×10^{-03}
AGAP2	504833	ArfGAP with GTPase domain, ankyrin repeat and PH domain 2	2.73	3.63×10^{-02}	3.09×10^{-02}
B3GNT6	540848	UDP-GlcNAc:betaGal beta-1,3-*N*-acetylglucosaminyltransferase 6	2.60	5.71×10^{-03}	6.62×10^{-03}
JAZF1	616701	JAZF zinc finger 1	2.60	5.71×10^{-03}	6.62×10^{-03}
RBFOX3	511773	RNA binding protein, fox-1 homolog 3	2.60	5.71×10^{-03}	6.62×10^{-03}
WDR27	788957	WD repeat domain 27	2.60	5.71×10^{-03}	6.62×10^{-03}
MNS1	532884	meiosis specific nuclear structural 1	2.56	9.60×10^{-04}	1.42×10^{-03}
TUBA8	768036	tubulin alpha 8	2.46	1.74×10^{-03}	2.39×10^{-03}

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Table 1. (*Continued.*)

Gene name	Gene ID	Gene description	$\log_2$ FC	*P* value	FDR
CD37	508751	CD37 molecule	2.46	1.06×10^{-02}	1.12×10^{-02}
CAP2	515190	CAP, adenylate cyclase-associated protein, 2 (yeast)	2.41	9.41×10^{-05}	1.86×10^{-04}
NHS	539895	NHS actin remodeling regulator	2.37	2.96×10^{-05}	6.52×10^{-05}
MUM1L1	539380	MUM1 like 1	2.31	9.53×10^{-04}	1.41×10^{-03}
ATP12A	530703	ATPase H+/K+ transporting non-gastric alpha2 subunit	2.31	1.95×10^{-02}	1.86×10^{-02}
FANCB	616549	Fanconi anemia complementation group B	2.31	1.95×10^{-02}	1.86×10^{-02}
TUBB1	541271	tubulin beta 1 class VI	2.31	1.95×10^{-02}	1.86×10^{-02}
ARL5B	616679	ADP ribosylation factor like GTPase 5B	2.14	2.60×10^{-06}	7.07×10^{-06}
PRRT3	504853	proline rich transmembrane protein 3	2.14	1.00×10^{-02}	1.07×10^{-02}
Downregulated					
COX7B	100300550	cytochrome c oxidase subunit VIIb	−8.19	2.73×10^{-33}	7.29×10^{-32}
HBA1	100140149	Hemoglobin subunit alpha	−7.52	8.77×10^{-23}	1.44×10^{-21}
CNTFR	539548	ciliary neurotrophic factor receptor	−5.61	1.32×10^{-07}	4.52×10^{-07}
MARC1	537028	mitochondrial amidoxime reducing component 1	−5.52	3.37×10^{-07}	1.08×10^{-06}
LOC 100336690	100336690	RAD51-associated protein 1	−4.94	3.47×10^{-05}	7.51×10^{-05}
Metazoa_SRP	102723303	Metazoan signal recognition particle RNA	−4.90	8.34×10^{-09}	3.42×10^{-08}
FGR	516133	FGR proto-oncogene, Src family tyrosine kinase	−4.44	6.51×10^{-04}	1.01×10^{-03}
LRRC23	613700	leucine rich repeat containing 23	−4.18	1.46×10^{-05}	3.42×10^{-05}
FSCN2	337926	fascin actin-bundling protein 2, retinal	−4.18	2.18×10^{-03}	2.90×10^{-03}
HIST1H1E	515957	histone cluster 1, H1e	−4.03	4.02×10^{-03}	4.91×10^{-03}
FRAT2	539144	FRAT regulator of WNT signaling pathway 2	−4.03	4.02×10^{-03}	4.91×10^{-03}
RBMS3	784766	RNA binding motif single stranded interacting protein 3	−3.94	8.56×10^{-05}	1.71×10^{-04}
TKTL1	507517	transketolase like 1	−3.91	6.24×10^{-03}	7.15×10^{-03}
SYT16	540677	synaptotagmin 16	−3.91	6.24×10^{-03}	7.15×10^{-03}
NPPC	281356	natriuretic peptide C	−3.86	7.47×10^{-03}	8.33×10^{-03}
SLC27A6	537062	solute carrier family 27 member 6	−3.86	7.47×10^{-03}	8.33×10^{-03}
IQCH	519277	IQ motif containing H	−3.86	7.47×10^{-03}	8.33×10^{-03}
RNF224	100336542	ring finger protein 224	−3.86	7.47×10^{-03}	8.33×10^{-03}
DCXR	782391	L-xylulose reductase-like	−3.86	7.47×10^{-03}	8.33×10^{-03}
DZIP1L	512800	DAZ interacting zinc finger protein 1 like	−3.66	1.40×10^{-02}	1.42×10^{-02}
LOC101906009	101906009	uncharacterized protein CXorf21 homolog	−3.66	1.40×10^{-02}	1.42×10^{-02}
PLVAP	524990	plasmalemma vesicle associated protein	−3.66	1.40×10^{-02}	1.42×10^{-02}
DOCK2	510083	dedicator of cytokinesis 2	−3.66	1.40×10^{-02}	1.42×10^{-02}
SOX30	538232	SRY-box 30	−3.56	9.23×10^{-04}	1.37×10^{-03}
LOC783616	783616	olfactory receptor 51L1	−3.44	1.68×10^{-03}	2.31×10^{-03}
ARL14EPL	616348	ADP ribosylation factor like GTPase 14 effector protein like	−3.44	2.63×10^{-02}	2.39×10^{-02}
FAM167A	618107	family with sequence similarity 167 member A	−3.44	2.63×10^{-02}	2.39×10^{-02}
DDX25	508962	ATP-dependent RNA helicase DDX25	−3.44	2.63×10^{-02}	2.39×10^{-02}
FHIT	692183	fragile histidine triad	−3.44	2.63×10^{-02}	2.39×10^{-02}
FAM71A	616153	family with sequence similarity 71 member A	−3.32	3.06×10^{-03}	3.88×10^{-03}
GPR183	540287	G protein-coupled receptor 183	−3.32	3.06×10^{-03}	3.88×10^{-03}
SFTPC	282071	surfactant protein C	−3.18	5.56×10^{-03}	6.48×10^{-03}
MYO1F	532964	myosin IF	−3.18	5.56×10^{-03}	6.48×10^{-03}

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Table 1. (*Continued.*)

Gene name	Gene ID	Gene description	$\log_2$ FC	*P* value	FDR
TMEM52	617403	transmembrane protein 52	−3.18	4.99×10^{-02}	4.03×10^{-02}
FBXO36	617339	F-box protein 36	−3.18	4.99×10^{-02}	4.03×10^{-02}
IFNE	101904725	interferon epsilon	−3.18	4.99×10^{-02}	4.03×10^{-02}
PADI2	617851	peptidyl arginine deiminase 2	−3.18	4.99×10^{-02}	4.03×10^{-02}
NTS	280881	Neurotensin	−3.18	4.99×10^{-02}	4.03×10^{-02}
CHGA	281070	chromogranin A	−3.03	2.77×10^{-04}	4.76×10^{-04}
SIGLEC1	539759	sialic acid binding Ig like lectin 1	−3.03	1.01×10^{-02}	1.08×10^{-02}
LRRC14B	100301203	leucine rich repeat containing 14B	−3.03	1.01×10^{-02}	1.08×10^{-02}
GIMAP8	531516	GTPase, IMAP family member 8	−2.86	8.60×10^{-04}	1.29×10^{-03}
HOXC13	538716	homeobox protein Hox-C13	−2.86	1.84×10^{-02}	1.78×10^{-02}
CNRIP1	539715	cannabinoid receptor interacting protein 1	−2.86	1.84×10^{-02}	1.78×10^{-02}
GUCA1A	282243	guanylate cyclase activator 1A	−2.86	1.84×10^{-02}	1.78×10^{-02}
FAM71F2	540061	family with sequence similarity 71 member F2	−2.86	1.84×10^{-02}	1.78×10^{-02}
C15H11orf96	100270684	chromosome 15 C11orf96 homolog	−2.86	1.84×10^{-02}	1.78×10^{-02}
NPY1R	504813	neuropeptide Y receptor Y1	−2.86	1.84×10^{-02}	1.78×10^{-02}
GSTA1	777644	glutathione S-transferase alpha 1	−2.76	1.51×10^{-03}	2.11×10^{-03}
SPACA6	113875604	sperm acrosome associated 6	−2.76	1.51×10^{-03}	2.11×10^{-03}

FC, fold change; FDR, false discovery rate.

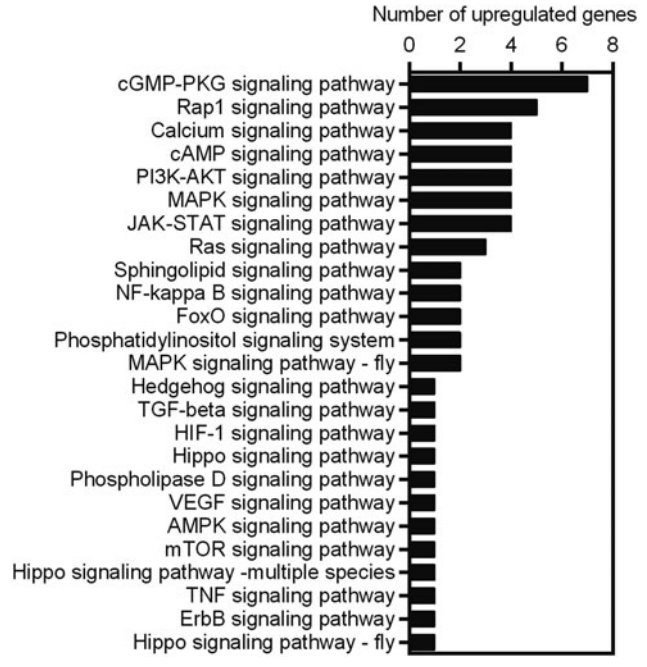

Fig. 1. Summary of KEGG pathways enriched in the signal transduction category. The *x* axis shows the number of upregulated genes for each term.

metabolic regulator that promotes glucose uptake in mouse 3T3 adipocytes. In dairy cows, plasma FGF21 increases at the onset of lactation (Schoenberg *et al.*, 2011). FGF21, betaKlotho, and fibroblast growth factor receptor 4 (FGFR4) form the cognate FGF21 receptor complex, which mediates FGF21 cellular specificity and physiological effects. Milk protein synthesis is an energetically costly process. The higher expression of *FGF21* and *FGFR4* in methionine-stimulated bovine mammary epithelial cells suggests enhanced glucose uptake and metabolism during methionine-induced milk protein synthesis in mammary glands of dairy cows.

In mice, the response of the mammary gland to prolactin, which leads to milk protein gene expression, is mediated by the JAK-STAT signaling pathway. Our data showed that the JAK-STAT signaling pathway was notably enriched for upregulated DEGs in methionine-stimulated cells. Four members of the JAK-STAT signaling pathway (*STAM*, *AKT3*, *GHR*, and *LIFR*) were significantly upregulated in methionine-stimulated mammary epithelial cells compared with control cells. Generally, growth hormone receptor (GHR) is expressed in the apical membranes of bovine mammary epithelial cells and mediates the function of growth hormone on milk protein gene expression (Zhou *et al.*, 2008). Our data suggested that the differential expression of these signaling molecules in JAK-STAT signaling was not hormone dependent but was mediated by methionine in bovine mammary epithelial cells.

Protein kinase B (PKB, also known as AKT) is an essential central regulator of mammary epithelial differentiation and lactation. Mice lacking *AKT1* exhibit a pronounced metabolic defect during late pregnancy and lactation. Our KEGG analysis revealed four upregulated DEGs in the PI3K-AKT signaling pathway. This is consistent with a recent study in dairy cow mammary gland showing that supplementation with methionine around parturition increases the phosphorylation of AKT, as well as the expression of amino acid transporters (Ma *et al.*, 2019). Studies of the roles of AKT in mice showed that loss of *AKT1*, but not *AKT2* or *AKT3*, inhibits milk production during lactation (Boxer *et al.*, 2006). However, our data showed that *AKT3* was

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upregulated by methionine stimulation, suggesting that *AKT3* may function in the regulation of milk protein synthesis in mammary glands of dairy cows. Additionally, we found that the PI3K-AKT and JAK-STAT signaling pathways were both significantly enriched for upregulated DEGs by methionine stimulation. This was in agreement with previous studies in mice showing that AKT is necessary for JAK-STAT5 activation and cell proliferation in lactating mammary gland (Chen *et al*., 2010).

The mammalian target of rapamycin is one of the downstream effectors of PI3K-AKT. Amino acid-induced protein synthesis is at least partially mediated by the mTOR signaling pathway. Several amino acids have been identified as effective activators of mTORC1. In the present study, we found only one upregulated gene related to the mTOR signaling pathway. In bovine mammary epithelial cells, phosphorylation of mTOR is significantly increased in methionine-stimulated cells compared with control cells, but the total level of mTOR does not change (Duan *et al*., 2017). This may indicate that methionine-regulated mTOR signaling is activated *via* a posttranscriptional mechanism in mammary glands of dairy cows.

In conclusion, we demonstrated that 458 transcripts (219 upregulated and 239 downregulated) were significantly differentially expressed between methionine-stimulated bovine mammary epithelial cells and control cells. Methionine-stimulated milk protein synthesis in bovine mammary epithelial cells is supported by increased gene expression related to the cGMP-PKG, Rap1, calcium, cAMP, PI3K-AKT, MAPK, and JAK-STAT signaling pathways. The identification of enriched DEGs and pathways provides insights into molecular events that occur in methionine-stimulated mammary epithelial cells. Understanding the regulatory effects of methionine on milk protein synthesis is important for improving the model of amino acid requirement for lactation.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029920000199.

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References

Appuhamy JA, Bell AL, Nayananjalie WA, Escobar J and Hanigan MD (2011) Essential amino acids regulate both initiation and elongation of mRNA translation independent of insulin in MAC-T cells and bovine mammary tissue slices. *Journal of Nutrition* **141**, 1209–1215.

Boxer RB, Stairs DB, Dugan KD, Notarfrancesco KL, Portocarrero CP, Keister BA, Belka GK, Cho H, Rathmell JC, Thompson CB, Birnbaum MJ and Chodosh LA (2006) Isoform-specific requirement for Akt1 in the developmental regulation of cellular metabolism during lactation. *Cell Metabolism* **4**, 475–490.

Chen CC, Boxer RB, Stairs DB, Portocarrero CP, Horton RH, Alvarez JV, Birnbaum MJ and Chodosh LA (2010) Akt is required for Stat5 activation and mammary differentiation. *Breast Cancer Research: BCR* **12**, R72.

Duan X, Lin Y, Lv H, Yang Y, Jiao H and Hou X (2017) Methionine induces LAT1 expression in dairy cow mammary gland by activating the mTORC1 signaling pathway. *DNA and Cell Biology* **36**, 1126–1133.

Hanigan MD, Crompton LA, Bequette BJ, Mills JA and France J (2002) Modelling mammary metabolism in the dairy cow to predict milk constituent yield, with emphasis on amino acid metabolism and milk protein production: model evaluation. *Journal of Theoretical Biology* **217**, 311–330.

Hicks MJ, Hu Q, Macrae E and DeWille J (2015) Mitogen-activated protein kinase signaling controls basal and oncostatin M-mediated JUNB gene expression. *Molecular and Cellular Biochemistry* **403**, 115–124.

Lin Y, Lv H, Jiang M, Zhou J, Song S and Hou X (2019) Functional analysis of the dairy cow mammary transcriptome between early lactation and mid-dry period. *Journal of Dairy Research*, 1–5.

Ma YF, Batistel F, Xu TL, Han LQ, Bucktrout R, Liang Y, Coleman DN, Parys C and Loor JJ (2019) Phosphorylation of AKT serine/threonine kinase and abundance of milk protein synthesis gene networks in mammary tissue in response to supply of methionine in periparturient Holstein cows. *Journal of Dairy Science* **102**, 4264–4274.

Schoenberg KM, Giesy SL, Harvatine KJ, Waldron MR, Cheng C, Kharitonenkov A and Boisclair YR (2011) Plasma FGF21 is elevated by the intense lipid mobilization of lactation. *Endocrinology* **152**, 4652–4661.

Zhou Y, Akers RM and Jiang H (2008) Growth hormone can induce expression of four major milk protein genes in transfected MAC-T cells. *Journal of Dairy Science* **91**, 100–108.