Animal Reproduction Science 208 (2019) 106135

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Identification of differential abundances of mRNA transcript in cumulus cells and CCND1 associated with yak oocyte developmental competence

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ARTICLE INFO

Keywords:
Yak
Cumulus cells
Oocyte quality
Expression
CCND1

ABSTRACT

The development of an accurate and noninvasive preselection process for competent oocytes is essential to achieve a highly efficient *in vitro* production (IVP) of embryos. Cumulus cells (CCs) have important functions in oocyte growth, development, maturation, and fertilization. It, therefore, is important to know if the quality of oocytes can be ascertained by assessment of gene expression of the surrounding CCs or not. The aim of this study was to identify differentially expressed genes in yak CCs from oocytes with varying developmental competences as possible biomarkers for distinguishing oocyte competence. The isolated CCs were pooled into immature and mature groups in accordance with the maturation outcome of oocytes. A total of 9516 genes were differentially expressed in the two CC categories ($P < 0.05$). With a minimum change of 2.5-fold, 45 up-regulated and 79 down-regulated genes were observed in CCs belonging to the mature group compared with those in the immature group ($P < 0.01$). These genes were primarily enriched for the cell cycle, meiosis, cell signaling, metabolism, and apoptosis. The selected candidate genes (*CCND1*, *BMP15*, *GDF9*, *H19*, *KLF4*, *GPC1*, *SYCP3,* and *CTSB*) were validated using quantitative real-time polymerase chain reaction (RT-qPCR) and there were expression patterns similar to those detected with transcriptome analysis. The CCs from fertilized oocytes arrested at the 2-cell (2-cell group), or 8-cell (8-cell group) stages or that developed into blastocysts (the blastocyst group) had a 1.5-, 1.8-, and 2.3-fold increase, respectively, in mRNA relative abundance of *CCND1* compared with CCs from unfertilized oocytes ($P < 0.05$). The results with the RT-qPCR analysis confirmed that the relative abundance of *CCND1* mRNA in CCs was associated with oocyte developmental competence. In conclusion, RNA-Seq is useful in extracting transcriptomes and selecting markers associated with oocyte developmental competence. Furthermore, the expression of the CCND1 gene in yak CCs can be used to preselect oocytes for IVP efficiency.

1. Introduction

A large number of competent oocytes are required for the *in vitro* production (IVP) of embryos and for basic research on mammals. At present, the recovery of immature oocytes followed by *in vitro* maturation (IVM) is a popular and effective approach to obtain

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https://doi.org/10.1016/j.anireprosci.2019.106135
Received 13 April 2019; Received in revised form 24 June 2019; Accepted 22 July 2019

mature oocytes for a wide range of applications, particularly in the assisted reproduction of mammals. With the IVM-derived oocytes, there is a greater risk of being compromised in terms of embryo development potential and quality than the *in vivo*-matured embryos (Banwell and Thompson, 2008). This is possibly due to *in vivo*-derived oocytes as a result of a long series of preparatory processes, whereas at the germinal vesicle (GV) stage there is inadequate cytoplasmic maturation as a result of the induced *in vitro* maturation after the shifting of oocytes from follicles to a culture medium. Thus, an indirect and noninvasive preselection system for the immature oocytes must be established to determine developmental competence before IVM.

At present, noninvasive oocyte selection is based mostly on morphological and developmental criteria (Scott, 2003; Kempisty et al., 2012). Morphological criteria, are not reliable for prediction of oocyte development or implantation capacity (Guerif et al., 2007; Rienzi et al., 2011). In recent years, methods for selecting of the immature oocytes have been developed to improve IVM efficacy, such as the use of the brilliant cresyl blue (BCB) staining protocol, which can be used as a noninvasive and indirect predictor of the underlying features of oocyte quality and developmental competence (Rienzi et al., 2011; Lonergan and Fair, 2016). Although this method allows for selection of oocytes with relatively greater maturation and cleavage rates from a heterogeneous pool, its potential toxicity to oocytes and developmental abnormalities subsequent to birth may be a concern when using this technique (Kątska-Książkiewicz et al., 2007; Opiela et al., 2010; Dutta et al., 2016). Even though there is a considerable amount of knowledge that has been obtained through IVM research, a more effective approach should be established to select the oocytes with the greatest capacity for producing blastocysts that when transferred result in a viable pregnancy.

The mammalian oocyte and its surrounding somatic cells are interdependent throughout the growth and development of the oocyte and the ovarian follicle. During follicular development and ovulation, oocytes are surrounded by cumulus cells (CCs). One of the major functions of CCs is channeling metabolites and nutrients, which are required for oocyte development, ovulation and fertilization to the oocyte. The CCs have important functions in acquiring oocyte competence during the maturation process (Tesfaye et al., 2009) and the developmental competence of an oocyte is adversely affected if CCs are removed before oocyte maturation occurs (Maedomari et al., 2007). The removal of oocytes from their natural inhibitory follicular environment results in the spontaneous resumption of meiosis that is independent of normal signaling events that occur *in vivo*. The assessment of the expression of CC marker genes as predictors of oocyte developmental competence has been recognized in recent years. Several genes expressed in CCs are important for oocyte maturation and development, and assessment of the expression of these genes can be a reliable method for selecting oocytes with the capacity to develop into a blastocyst and ultimately result in birth of live offspring (Russell and Robker, 2007).

Recent developments in RNA-Seq technology have provided a global transcriptomic approach for identifying differentially expressed genes that are important for oocyte maturity. The RNA-Seq analyses have focused on early embryo development or implantation capacity. The aim of the current study was to investigate temporal changes in the expression of CC genes during oocyte maturation as possible biomarkers for oocyte developmental competence in yak. The temporal expression patterns of the CC expressed genes were hypothesized to differ with relatively greater compared to lesser quality yak oocytes.

2. Material and methods

All the chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise noted. Disposable, sterile plasticware was purchased from Nunclon (Roskilde, Denmark). All procedures in this experiment were approved by the Animal Care and Use Committee of Southwest Minzu University and performed in accordance with animal welfare and ethics.

2.1. Ovary collection and IVM

Yak ovaries were collected from a local abattoir (Chengdu, Sichuan, China), stored in a thermos bottle with sterile physiological saline (supplemented with 100 IU/mL penicillin and 80 IU/mL streptomycin) at 30 °C, and then transported to the laboratory within 2 h. The COCs were collected from ovaries using a 12-gauge needle attached to a 10 mL syringe with 1 mL phosphate buffered saline (PBS). The COCs with a homogeneous cytoplasm and surrounded by more than three layers of compact CCs were selected for IVM. Depending on the experimental design, several CCs were collected from the COCs using a mechanical separation method, by repeated pipetting before IVM. The CCs were stored at -80 °C until RNA isolation and the subsequent sequencing analysis. The corresponding COC was cultured individually in a droplet with IVM medium (TCM199 supplemented with 10% FBS, 1 μg/mL 17β-estradiol, 0.5 μg/mL LH and 0.5 μg/mL FSH) that were covered with mineral oil at 38.5 °C in a 5% CO_2 atmosphere in a CO_2 incubator for 24 h.

In accordance with the maturation outcome of the oocytes, the previously isolated CCs were pooled into two groups before RNA extraction: those from oocytes that failed to extrude the first polar body (the immature group) and those from oocytes that extruded the first polar body (the mature group). For each group, three replicas of pooled CCs that corresponded to 30 oocytes were used for the subsequent RNA-Seq analyses, and three independent replicates corresponding to 50 oocytes were used for RT-qPCR.

2.2. RNA isolation and illumina-solexa sequencing

The total RNA of CCs was extracted using RNeasy Plus Micro Kit (Qiagen, Germany) following to the manufacturer's instructions. The Poly (A) mRNA was isolated from the RNA pool and purified using Oligotex mRNA Midi Kit (Qiagen, Germany). The quality and quantity of the purified RNA were determined by measuring absorbance at 260 nm/280 nm using Nanodrop ND-1000 spectrophotometer (LabTech, USA). The RNA integrity was evaluated using electrophoresis utilizing a 1.5% (w/v) agarose gel. The random fragment sequencing library was built using SOLiD Whole Transcriptome Analysis Kit (Life technologies, USA) following to the manufacturer's standard processes. After conducting quality control tests with Agilent 2100 Bioanalyzer and ABI Step One Plus Real-Time PCR System, the library was sequenced using an Illumina HiSeq™ 2000.

2.3. Transcriptome data analysis

Transcriptome data were analyzed on the basis of the standard program and results from a previous study (Lan et al., 2014). To compare differential abundances of mRNA transcript between immature and mature groups, the read count of each identified mRNA was normalized to the total number of mRNA read counts that matched the yak genome in each sample, and the relative abundance of various mRNA families was determined by calculating the sequencing frequency as described in previous studies (Wanger et al., 2012). A minimum change of ± 1.5 -fold and $P < 0.05$ were used as a threshold to classify the differentially expressed genes in this study. The biological functions and involved pathways of these genes differed between the two groups and were analyzed using the WEGO program and the Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.4. In vitro fertilization (IVF) and in vitro culture (IVC)

After maturation, the oocytes were cultured individually for IVF and IVC as described in a previous study (Xiong et al., 2017). After maturation, COCs were transferred to the respective microdrop (10 μL) of Fert-Talp medium supplemented with 5 g/L heparin sodium, 1.6 g/L hypotaurine, 0.3 g/L epinephrine, and 100 IU/mL penicillamine. Each drop was covered with mineral oil and pre-incubated for at least 2 h at 38.5 °C in a CO_2 incubator. Subsequently, the prepared spermatozoa were added to each drop and the mixture was incubated for approximately 16 h. Presumptive zygotes were denuded by treating with 0.2% bovine testicular hyaluronidase in PBS, washed twice in mSOF, and then cultured in drops of the sequential culture medium G1 (Vitrolife, Sweden). After 96 h post-culture, the embryos were transferred to G2 drops to continue incubation. Subsequent *in vitro* development to the 2-cell, 8-cell, and blastocyst stages was monitored at 24, 72, and 168 h, respectively.

2.5. RT-qPCR

The CCs were pooled into four groups before RNA extraction: CCs from oocytes that failed to fertilize (the unfertilized group), CCs from oocytes that were fertilized and arrested at the 2-cell stage (the 2-cell group), CCs from oocytes that were arrested at the 8-cell stage (the 8-cell group), and CCs from oocytes that developed into blastocysts (the blastocyst group). Total RNA isolation and purification, *in vitro* transcription, and RT-qPCR analyses were performed as described in previous studies (Xiong et al., 2017; Bustin et al., 2009) to quantify the abundances of mRNA for cyclin D1 (*CCND1*), bone morphogenetic protein 15 (*BMP15*), growth differentiation factor 9 (*GDF9*), *H19*, kruppel like factor 4 (*KLF4*), glypican 1 (*GPC1*), synaptonemal complex protein 3 (*SYCP3*), and cathepsin B (*CTSB*). The RNA extraction was performed for each group using Cells-to-Signal™ Kit (Invitrogen, USA) following the protocol of the manufacturer. The extracted RNA was immediately used for reverse transcription utilizing a cDNA synthesis kit (Takara, Dalian, China) following the manufacturer's instructions. The PCR primer sequences were designed cross-intron using Primer 5.0 software as shown in Table 1. The relative fold change of genes was calculated using the $2^{-\triangle\triangle Ct}$ method as described in Xiong et al. (2012), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the endogenous reference gene. Each sample was prepared independently in triplicate. The average mRNA abundance of each gene from the control group was set as 1 for easy comparison.

Table 1
Primers sequences and PCR conditions for analysis of gene expression.

Gene	Primer sequences (5'-3')	Tm (ºC)	Product size (bp)	Accession no.
BMP15	ACTTCACCTAACTCATTCCCACCTC TGCCACCAGAACTCACGAACC	55	249	XM_005898488
CCND1	CCTGGTGAACAAACTCAAGTGGAAC GGCACAGAGGGCAACGAAGGT	58	142	KY_420723
GDF9	CTGAGACTTGGTCCTTGCTGA GGTGGCTTCTGTTGGATTTAG	54	198	EU_267798
H19	CTGGTCAGGCAGCACAGGTGA AGTGAATTACAGTGGGTGAAGTGGC	60	244	NR_003958
KLF4	TGAACTGACCAGGCACTACCG TTGACTTGTTGGGAACTTGACC	55	253	NM_001105385
GPC1	ATCAACGCCCTCCAGGACAACAG GCCAAGCCATCACCCATCACC	61	245	XM_014480020
SYCP3	CTATAAGAGCCTATGAATTTGAGC CAGCATATTCTGTACTTCACCC	50	158	XM_014479077
CTSB	CCTTTGGAGAAAGCCCTGTTGT ACCGAGGTCAGTCAAGTCACAACC	55	212	XM_005886688
GAPDH	TGCTGGTGCTGAGTAGTTGGT TCTTCTGGGTGGCAGTGATGG	58	292	AC_000162

2.6. Relationship between CCND1 expression and oocyte development

To further analyze the relationship between the abundance of CCND1 mRNA transcript and the developmental potential of oocytes, *CCND1* mRNA in CCs from each COC was detected *via* RT-qPCR. Depending on the relative mRNA abundance, the corresponding COCs were pooled into the relatively lesser-expression group (Lesser Expression Group) and greater-expression groups (Greater Expression Group) for IVM. After maturation, the oocytes were used for IVF and IVC, as previously described in this manuscript, and for the development of presumptive zygotes.

2.7. Statistical analysis

The experiment was repeated at least three times for each treatment group. Each replicate was performed using oocytes that matured on the same day to liminate any batch effect of oocytes. The cleavage, 8-cell, and blastocysts formation rates and the relative abundances of mRNA transcripts were assessed among the groups using a one-way analysis of variance (ANOVA) and least significant difference test using SPSS 13.0 software. Data are presented as mean ± SEM unless indicated otherwise. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Transcriptome profiles of CCs

Results from the RNA-Seq experiments indicated there were similar relative abundance patterns for mRNA between the immature and mature oocyte groups. A small number of genes encoding for transcripts with varying mRNA abundances in CCs surrounded GV stage oocytes, which were either mature or immature. The results from the transcriptome analysis resulted in there being 9516 differentially expressed genes. Among which 3806 genes encoding for transcripts that were of a greater mRNA abundance in CCs, these CCs surrounded oocytes that advanced to the mature developmental stage. Furthermore, there were 5710 genes encoding for transcripts that were of greater mRNA abundance in CCs surrounding oocytes that failed to mature ($P < 0.05$). Several differentially expressed genes (including 45 upregulated and 79 downregulated) with a change ≥ ± 2.5-fold are listed in Table 2. Among these genes, 119 can be categorized as being involved in one or more biological processes, primarily in the cell cycle (26), meiosis (21), cell signaling (18), metabolism (17), apoptosis (13), and others (24), as shown in Fig. 1.

3.2. Validation of RNA-Seq data via RT-qPCR

The genes involved in cell cycle and meiosis were overrepresented in the CC samples harvested from oocytes that developed to the mature stage. With a change ≥ ± 2.5-fold, four up-regulated genes (*CCND1*, *BMP15*, *GDF9* and *H19*) and four down-regulated genes (*KLF4*, *GPC1*, *SYCP3* and *CTSB*) were selected as candidates for biomarkers to determine the functional relationship to oocyte developmental competence. The mRNA was verified in CCs using RT-qPCR relative to the expression of the GAPDH gene (Fig.2). Among the selected genes, there was no difference in relative abundance of KLF4 and H19 mRNA in CCs from oocytes that were in the immature and mature groups. By contrast, the results for the other genes assessed using RT-qPCR are consistent with results of the RNA-Seq data.

3.3. Relative abundance of CCND1 mRNA in yak CCs

The RT-qPCR procedures were used to detect the relative abundance of CCND1 mRNA in CCs from different developmental competent oocytes (Fig. 3). Compared with CCs from unfertilized oocytes, those from fertilized oocytes had a 1.5- to 2.3-fold greater relative abundance of CCND1 mRNA. To further assess the possible correlation between CCND1 gene expression in CCs and the development potential of oocytes, oocytes were divided into three groups after fertilization and cleavage: the 2-cell (embryos arrested at the 2-cell stage), 8-cell (embryos arrested at the 8-cell stage), and blastocyst-group (embryos developed into the blastocyst stage). The relative abundance of *CCND1* mRNA in CCs from oocytes that after fertilization developed into blastocyst stage embryos was 1.3 times greater than that in CCs from oocytes arrested at the 8-cell developmental stage, and 2.3 times greater ($P < 0.01$) than that of CCs from unfertilized oocytes.

3.4. Correlation between CCND1 mRNA abundance and oocyte quality

We hypothesized that the abundance of *CCND1* mRNA in CCs is positively correlated with yak oocyte quality. To test this hypothesis, *CCND1* mRNA in CCs from each COC was detected using RT-qPCR. Based on the mRNA abundances, the corresponding COCs were pooled into two groups for IVM: Relative mRNA abundance (*CCND1/GAPDH*) < 1.5 (Lesser Expression Group) and the relative mRNA abundance (*CCND1/GAPDH*) ≥ 1.5 (Greater Expression Group). The IVF procedures were subsequently performed to measure the quality of an oocyte as assessed by its potential to develop into a blastocyst and its relationship with CCND1 mRNA abundance (Table 3). After 24 h of IVM, the maturation rate was less ($P < 0.05$) in the Lesser Expression Group (54.7%) compared with the Greater Expression Group (86.3%). The relative abundance of *CCND1* in CCs achieved higher developmental competence for oocytes, although the cleavage rates were not significantly different ($P > 0.05$). The oocytes harvested from the *CCND1* Greater

Table 2
Part of differential genes expression in CCs between mature group and immature group.

Gene name	Fold change	Expression*	Gene name	Fold change	Expression*
Lumican (LUM)	7.8	Up	Prostaglandin-endoperoxide synthase 2 (PTGS2)	3.5	Down
Interleukin 33 (IL33)	7.5	Up	Homeobox A10 (HOXA10)	3.5	Down
Cyclin D1 (CCND1)	7.0	Up	Calicin (CCIN)	3.4	Down
Melatonin receptor 1B (MTNR1B)	6.8	Up	Equilibrative nucleoside transporter 2 (Ent2)	3.4	Down
Cadherin-2 (CDH2)	6.7	Up	Chromogranin-a (Chga)	3.4	Down
Neuronal nicotinic acetylcholine receptor beta4 subunit (CHRNB4)	6.6	Up	Prostaglandin I2 (prostacyclin) synthase (PTGIS)	3.4	Down
Tumor necrosis factor, alpha induced protein 6 (TNFAIP6)	6.5	Up	Secreted frizzled-related protein 2 (SFRP2)	3.4	Down
Zinc finger E-box binding homeobox 2 (ZEB2)	6.4	Up	DAZ associated protein 1 (DAZAP1)	3.3	Down
ISG15 ubiquitin-like modifier (ISG15)	6.0	Up	Interleukin-18 (Il18)	3.3	Down
MX dynamin like gtpase 1 (MX1)	5.5	Up	Killin (Klln)	3.3	Down
Galectin 3 binding protein (LGALS3BP)	5.5	Up	Glia maturation factor gamma (GMFG)	3.3	Down
2'-5'-oligoadenylate synthase 3-like (LOC102283231)	4.9	Up	Neurofascin (Nfasc)	3.3	Down
Basic helix-loop-helix family member e40 (BHLHE40)	4.8	Up	Sphingomyelin phosphodiesterase 1 (SMPD1)	3.3	Down
Microtubule-associated proteins 1A/1B light chain 3C (mlp3c)	4.6	Up	Haptoglobin (Hp)	3.3	Down
Bone morphogenetic protein 15 (BMP15)	4.4	Up	Fumarylacetoacetase (Fah)	3.2	Down
Interferon, alpha inducible protein 27-like 2 (IFI27L2)	4.2	Up	Transmembrane and coiled-coil domain-containing protein 4	3.2	Down
GTP-binding protein (GEM)	4.1	Up	Mediator of RNA polymerase II transcription subunit 25	3.2	Down
Growth differentiation factor 9 (GDF9)	4.0	Up	Synaptonemal complex protein 3 (SYCP3)	3.2	Down
Integrin, alpha 6 (ITGA6)	3.9	Up	ATP synthase lipid-binding protein, mitochondrial	3.2	Down
Solute carrier family 2 facilitated glucose transporter member 3 SLC2A3P1)	3.8	Up	Sperm associated antigen 4 (SPAG4)	3.2	Down
Follistatin (FST)	3.7	Up	Vascular endothelial growth factor A (VEGFA)	3.2	Down
Stanniocalcin-1 (Stc1)	3.7	Up	Mevalonate decarboxylase (MVD)	3.2	Down
Galectin 3 binding protein (LGALS3BP)	3.6	Up	Integrin subunit alpha 6 (ITGA6)	3.2	Down
Secreted protein acidic and cysteine rich (SPARC)	3.2	Up	Sorbitol dehydrogenase (SORD)	3.2	Down
Tetraspanin-6 (Tspan6)	3.1	Up	Argininosuccinate lyase (Asl)	3.2	Down
H19	3.0	Up	Regulator of G-protein signaling 1 (Rgs1)	3.2	Down
Retinoic acid receptor (RAR)	3.0	Up	Vascular endothelial growth factor B (VEGFB)	3.1	Down
Aquaporin-3 (aqp3)	2.9	Up	Arginase 2 (ARG2)	3.1	Down
SPARC-like protein 1 (SPARCL1)	2.9	Up	Galactokinase 1 (GALK1)	3.1	Down
Phospholipid scramblase 1 (PLSCR2)	2.9	Up	ZFP36 ring finger protein (ZFP36)	3.1	Down
Pentraxin 3 (PTX3)	2.8	Up	Pleiotrophin (PTN)	3.1	Down
Milk fat globule-EGF factor 8 protein (MFGE8)	2.8	Up	Stabilin 1 (Stab1)	3.1	Down
Stimulated by retinoic acid gene 6 protein-like (LOC109563057)	2.8	Up	Cadherin 8 (CDH8)	3.1	Down
Melatonin receptor 1a (MTNR1A)	2.7	Up	Phosphomannomutase (PMM)	3.1	Down
Phosphoglycerate dehydrogenase (PHGDH)	2.7	Up	Calreticulin-3 (Crt3)	3.1	Down
Inhibin subunit alpha (INHA)	2.7	Up	Bostaurusegl nine homolog 3 (EGLN3)	3.1	Down
Semaphorin 3C (SEMA3C)	2.7	Up	Solute carrier family 1 member 1 (SLC1A1)	3.0	Down
Hyaluronan synthase 2	2.6	Up	Lactate dehydrogenase C (LDHC)	3.0	Down
Hyaluronan synthase 2 (HAS2)	2.6	Up	Erythropoietin (EPO)	3.0	Down
Lactate dehydrogenase A (LDHA)	2.6	Up	KIT proto-oncogene receptor tyrosine kinase (KIT)	3.0	Down
Sec23 homolog A (SEC23A)	2.5	Up	Immediate early response 3 (IER3)	3.0	Down
MAGE family member D2 (MAGED2)	2.5	Up	Growth hormone receptor (GHR)	3.0	Down
ADP ribosylation factor like GTpase 13B (ARL13B)	2.5	Up	6-phosphogluconolactonase (6pgle)	3.0	Down
Methionine adenosyltransferase 2A (MAT2A)	2.5	Up	Glypican 1 (GPC1)	3.0	Down
Fibroblast growth factor 11 (FGF11)	2.5	Up	Regulator of G-protein signaling 2 (Rgs2)	3.0	Down
BOP1 ribosomal biogenesis factor (BOP1)	5.1	Down	Serpinf1	3.0	Down
Homeobox D4 (HOXD4)	4.9	Down	Serum response factor (SRF)	2.9	Down
Perilipin 5 (PLIN5)	4.8	Down	Kruppel-like factor 10 (KLF10)	2.9	Down
Cytohesin 2 (CYTH2)	4.2	Down	Heat shock transcription factor 2 binding protein (HSF2BP)	2.9	Down
Spermidine synthase (SRM)	4.2	Down	BCL2 associated X, apoptosis regulator (BAX)	2.9	Down
Cathepsin B (CTSB)	4.1	Down	DEAD-box helicase 25 (DDX25)	2.9	Down
Eukaryotictranslation initiation factor 3 (Eif3cl)	3.7	Down	G protein-coupled receptor kinase 5 (GRK5)	2.9	Down
Follicle stimulating hormone receptor (FSHR)	3.6	Down	Cyclin I family member 2 (CCNI2)	2.9	Down
Transducin beta like 1 X-linked (TBL1X)	3.6	Down	Lutaredoxin 3 (Glrx3)	2.9	Down
Taperin (Tprn)	3.6	Down	Nucleoredoxin (NXN)	2.9	Down

(*continued on next page*)

Table 2 (*continued*)

Gene name	Fold change	Expression*	Gene name	Fold change	Expression*
Kinesin family member 19 (KIF19)	3.6	Down	Troponin c (Tnc)	2.9	Down
Tectonin beta-propeller repeat containing 1 (TECPR1)	3.6	Down	Keratin (Kap6)	2.8	Down
Glycine amidinotransferase (GATM)	3.6	Down	Nitric oxide synthase interacting protein (NOSIP)	2.8	Down
Mannosidase, alpha, class 1A, member 1(MAN1A1)	3.6	Down	Fibrillin 3 (FBN3)	2.7	Down
Heat shock 70kda protein 2 (HSPA2)	3.5	Down	Jagged 2 (JAG2)	2.7	Down
Integrin alpha 6 (ITGA6)	3.5	Down	Interleukin 4 receptor (IL4R)	2.6	Down
Mortality factor 4 like 2 (MORF4L2)	3.5	Down	Glycine N-methyltransferase (GNMT)	2.6	Down
Isoleucyl-trna synthetase 2 (IARS2)	3.5	Down	Kruppel-like factor 4 (KLF4)	2.5	Down

* Expression status of differentially expressed genes in this study, using the CCs from immature group as reference, the fold changes represent mRNA abundant in mature group compared to immature counterparts.

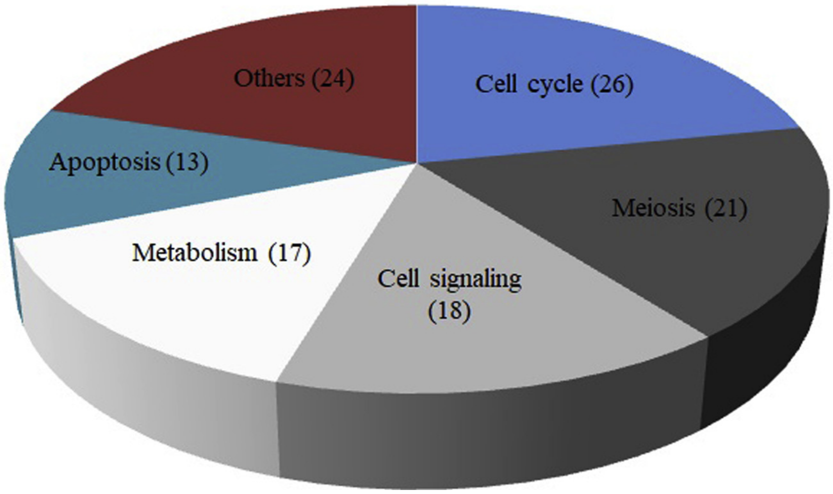

Fig. 1. Functional classification of genes with differential abundances of mRNA transcript using the KEGG database.

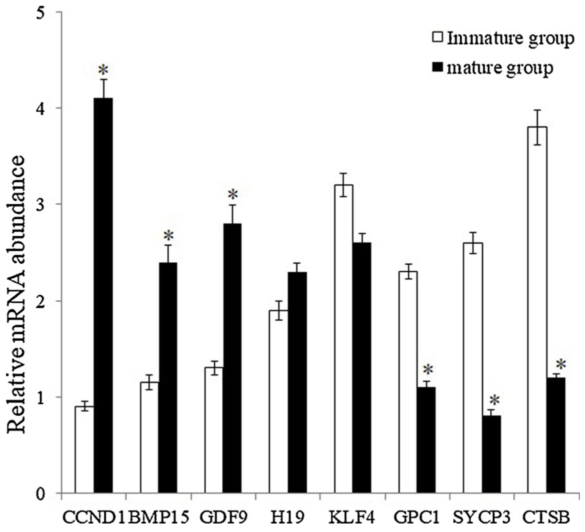

Fig. 2. Validation of the genes with differential patterns of mRNA transcript abundances in CCs using RT-qPCR. Data are mean ± SEM ($n = 3$), and asterisk (*) within each graph indicates differences ($P < 0.05$).

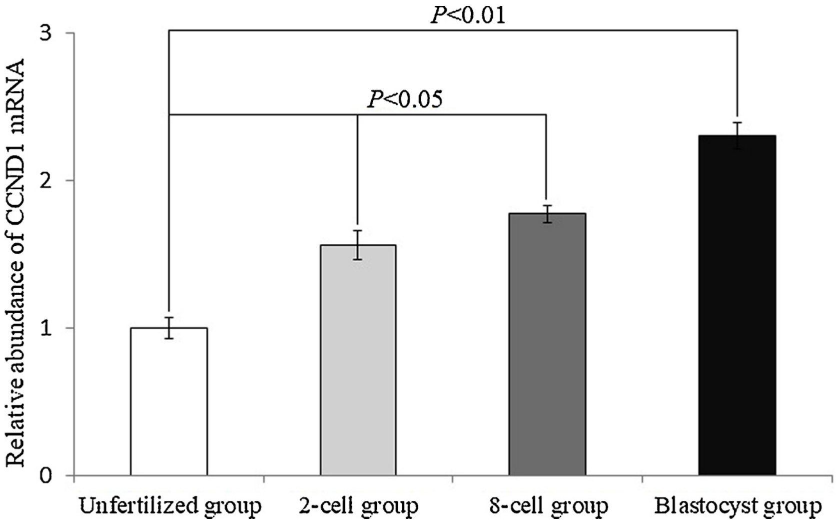

Fig. 3. Relative abundance of *CCND1* mRNA transcript in CCs of oocytes with different developmental competence; Data are mean ± SEM ($n = 3$), oocytes were divided into three groups after fertilization and cleavage: 2-cell group (embryos arrested at the 2-cell stage), 8-cell group (embryos arrested at the 8-cell stage), and blastocyst group (embryos developing to the blastocyst stage).

Table 3
The effects of *CCND1* expression on the developmental potential of oocyte.

Group	No. of COCs	No. of matured (%)	No. of 2-cell (%)	No. of 8-cell (%)	No. of blastocyst(%)
Low	75(4)	41(54.7 ± 3.3)^{a}	30(73.2 ± 2.8)^{a}	16(53.3 ± 2.9)^{a}	7(23.3 ± 3.5)^{a}
High	102(4)	88(86.3 ± 1.9)^{b}	71(80.7 ± 2.2)^{a}	50(70.4 ± 1.7)^{b}	36(50.7 ± 2.6)^{b}

Low group: the relative expression (*CCND1/GAPDH*) < 1.5, High group: the relative expression (*CCND1/GAPDH*) ≥ 1.5. Values with dissimilar superscripts (a–c) in the same column were significantly different ($P < 0.05$). Four replicates.

Expression Group had greater rates of embryo development to the 8-cell stage and for blastocyst formation than those from the *CCND1* Lesser Expression Group ($P < 0.05$).

4. Discussion

Although the use of microarrays to explore complex transcriptional profiles is increasing, there are needs for a further design of probes and there needs to be a large number of cells to utilize for the use of microarrays. It, therefore, is not possible to use microarrays for assessment of transcriptional profiles in specialized cells such as oocytes (Werner, 2008; Tariq et al., 2011). As an alternative approach, RNA-Seq has been recently utilized as a next-generation sequencing technology. This technique appears to provide more precise characterization of RNA transcripts (Mortazavi et al., 2008; Wang et al., 2009). In the present study, RNA-Seq was used to obtain a global profile of gene expression in CCs and to correlate the gene expression patterns with the developmental status of oocytes surrounded by these CCs.

The use of IVP and assisted reproductive technologies have increased, but the efficiency of these methods remains inadequate. Understanding the mechanisms and pathways that underlie embryo developmental competence, therefore, remains important. Given the essential nature of the interaction between CCs and a maturing oocyte (Regassa et al., 2011), the analysis of CCs appears to be a logical potential approach for noninvasive assessment of oocyte developmental competence (Lédée et al., 2010). The CCs have important functions in the oocyte development process, maintenance of meiotic arrest, and cytoplasmic and nuclear maturation of oocytes (Feuerstein et al., 2007; Tesfaye et al., 2009). This interdependence between oocytes and CCs can allow for an indirect and noninvasive evaluation of oocyte competence prior to IVP (Ekart et al., 2013; Iager et al., 2013; Bunel et al., 2015). The aim of the present study was to investigate the gene expression profiles of CCs derived from yak COCs with different developmental potentials. The results indicated that the CC gene expression profile of the immature group was distinctly different from that of the mature group. The results of the KEGG analysis allowed for differentially expressed genes to be detected that are mostly involved in the cell cycle and meiosis pathways. This finding is consistent with the results of Melo et al. (2016). Along with the growth and maturation of follicles, CCs that surrounded oocytes rapidly proliferate and divide. This process requires an intense network signaling to facilitate cell division and differentiation (Assidi et al., 2010). The genes involved in cell cycle, meiosis, metabolism, and apoptosis have an increased expression when the cellular status needs to be regulated by activation of one or more of these cell functions.

The expression profiles of eight selected genes were investigated in CCs derived from different developmentally competent oocytes, including the up-regulated *CCND1*, *BMP15*, *GDF9* and *H19* and down-regulated *KLF4*, *GPC1*, *SYCP3* and *CTSB* genes. The expression profiles of most of these genes have been used as physiological markers of oocyte competence and quality in previous studies (Gebhardt et al., 2011; Xu et al., 2015). The GDF9 and BMP15 proteins have important functions in cumulus expansion and oocyte maturation (Zhang et al., 2005). For example, GDF9 affects the cumulus oophorus formation and oocyte fertilization rates by regulating the expression of FSH-induced steroidogenesis, LH receptor numbers, pentraxin-3 (Ptx3), and other genes in CCs (Varani et al., 2002; Zhang et al., 2005). Nevertheless, Bettegowda et al. (2008) reported that there was a robust negative relationship between *CTSB* mRNA abundance and oocyte competence and suggested that *CTSB* gene expression may be functionally coupled to oocyte competence (Bettegowda et al., 2008). Van Montfoort et al. (2008) reported that there were many differentially expressed genes among various oocytes, and a number of these genes are involved in the cell cycle, such as CCND and glutathione peroxidase3. The altered expressions of these genes were suggested to affect oocyte maturation or the subsequent embryo development. The *GPC1* gene is a member of the glypican family, and it mediates the expression of cumulus-specific matrix genes *HAS2* and *TNFAIP6* (Watson et al., 2012). In the present study, all the selected gene groups belong to the cell cycle and meiosis categories, among which, four are upregulated genes and four are downregulated genes. Data from the present study indicate that there is expression of cell cycle related genes in CCs from oocytes that have lesser developmental competence, but there is up-regulation of the *H19* gene in CCs surrounding immature oocytes. These transcriptional profiles may be explained by an increased requirement for oocyte growth in preparation for ovulation, although there was no significant difference detected in these transcriptional profiles for these genes when there was assessment using RT-qPCR. In contrast, for the SYCP3 meiosis category gene there was an apparent increase in expression of this gene in developmentally competent oocytes, however, in the present study the data were contrasting in this regard. Even though there has been a large number of studies of the SYCP gene family, there is no information about the function of the SYCP3 protein in oocyte growth and development. Further investigation is necessary to determine the function of the SYCP3 protein in oocyte competence and its potential use as a genetic marker.

Evidence accumulated from several studies indicates the CCs have important functions during oogenesis and oocyte development. The analysis of the cumulus transcriptomes in relation to oocyte maturation and developmental competence may help identify novel biomarkers as alternatives to conventional morphological criteria.

Various markers of oocyte quality have been assessed for usefulness in predicting the developmental potential of oocytes. A greater mRNA abundance has been reported for HAS2, PTGS2, and GREM1 in CCs surrounding oocytes that contribute to production of high-grade embryos (McKenzie et al., 2004), and there is a positive association between increased CC Ptx3 mRNA abundance with successful IVF and the developmental/implantation potential of human embryos *in vitro* (Zhang et al., 2005). Results of previous studies indicate CCND1 is involved in germinal vesicle breakdown (GVBD) and meiotic maturation of mammalian oocytes (Anguita et al., 2008), and expression of the CCND1 gene has been reported in immature and mature oocytes in different mammalian species (Kohoutek et al., 2004; Kuroda et al., 2004). In the current study, the CCND1 gene was expressed in CCs from different developmentally competent oocytes (Figs. 2 and 3). Considering CCND1 is essential for oocyte development and meiotic maturation, the notion that this gene is expressed throughout the process of oocyte maturation in oocytes and CCs is conceivable. Results from the present study indicate there is a sustained increase in the expression of the CCND1 gene in CCs that surround oocytes regardless of the oocyte quality. The expression of the CCND1 gene, therefore, is closely related to the developmental potential of oocytes.

To further verify the relevance between the two variables, oocytes were divided into two groups based on extent of CCND1 gene expression. A greater relative abundance of *CCND1* mRNA transcript in CCs was associated with a greater developmental competence of oocytes (Fig. 3). Cooper et al. (2015) reported that CCND1 is an important factor in the multiplication and expansion of CCs, regulation of GVBD, and maturation of oocytes (Robert et al., 2002). Furthermore, the reduced expression of the CCND1 gene in CCs at different developmental stages of corresponding oocytes may possibly compromise the developmental potential of oocytes (Praveen Chakravarthi et al., 2016). Consistent with the finding of Praveen Chakravarthi et al. (2016), results of the present study indicate there were differences and a positive correlation in CCND1 gene expression in CCs that surround oocytes with different developmental potentials. The subtle changes in the expression of the CCND1 gene are believed to precede gross development abnormalities, and such an inference should be supported in other mammal species. Future studies on temporal changes in the expression of the CCND1 gene in CCs should provide more substantial proof for this association.

In conclusion, results from the present study provide evidence for the marked differences in the CC gene expression profiles among yak oocytes with differential developmental competence. The results of this study support a positive association and a potential functional relationship between the relative abundance of *CCND1* mRNA of CCs and oocyte competence. The results also suggest that the transcript abundance of CCND1 in CCs may provide a novel promising approach for preselecting yak oocytes and predicting the capacity to support development toward the blastocyst stage after fertilization. Although further studies on implantation and post-implantation developmental competence are required, the evaluation of oocyte developmental competence on the basis of the *CCND1* mRNA abundance in surrounding CCs can be used as an efficient and noninvasive preselection method without using additional chemicals or equipment.

Declaration of Competing Interest

We state that the authors or author’s institution have no financial or other relationship with other people or organizations that may inappropriately influence the author’s work.

Acknowledgements

The National Key Research and Development Program (2018YFD0502304), Innovation Team Project for Conservation and

Utilization of Yak Genetic Resources (13CXTD01), the Fundamental Research Funds for the Central Universities of Southwest Minzu University (2019NQN45) and Collaborative Innovation Center of Qinghai-Tibet Plateau Ecological Animal Husbandry (QZGYXT05) supported the present study.

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