Identification of Differentially Expressed Long Noncoding RNAs and mRNAs Involved with Dominant Follicle Selection in Goats using RNA-seq

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ABSTRACT

In this study, we used high-throughput technology to provide the first transcriptome dataset for differentially expressed genes in mixed pools of dominant and nondominant follicles of goats. These data will contribute to research on the molecular mechanisms of dominant follicle selection in goats. In this study, 90276370 and 115579236 clear reads in dominant and nondominant follicles of goat were generated through Illumina paired-end sequencing, and their mapping rate was 84.99% and 84.47%, respectively. A total of 12577 differentially expressed genes (DEGs) were identified, including 6009 upregulated and 6568 downregulated genes in dominant follicles compared with nondominant follicles of goat. Of the 1026 significantly differentially expressed, long noncoding RNAs (lncRNAs) found, 419 were upregulated and 607 were downregulated. The DEGs related to 56 GO categories, and pathway analysis revealed that these DEGs were significantly enriched in 41 of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, including signaling pathways regulating the pluripotency of stem cells, the p53 signaling pathway, and oxidative phosphorylation. The results of the present study confirmed that the selection of the dominant follicle involved the regulation of various physiological systems. These results provided helpful data to understand on the molecular mechanisms of dominant follicle selection in goats.

INTRODUCTION

Recent advances in high-throughput technology have led to the demonstration that lncRNA is actively transcribed in the eukaryotic genome and plays an important role in all aspects of normal biological function, particularly in model animals (Yue *et al.*, 2016).

Follicle selection, the process giving rise to the dominant follicle (DF), and the physiological state of the DF are important processes to study, especially the selection of an ovarian follicle for further differentiation and finally ovulation in an ovulating animal. However,

the transcriptional regulation of ovarian follicles is very complex, and large differences have been observed during different developmental stages and in different breeds (Zhao *et al*., 2015). Therefore, the precise mechanisms regulating the growth of follicles and ovulation are still poorly understood.

In this study, we investigated the differential expression of lncRNAs and mRNAs in different development periods of follicles using RNA-seq technology. The data provide a large amount of useful information about RNAs that are related to mammal reproductive processes, and they help to understand the importance of different transcriptional factors in follicular development.

MATERIALS AND METHODS

Animals

The experimental conditions of this study were approved by the Committee on the Ethics of Animal Experiments of the Southwest University (No. [2007] 3) and the Animal Protection Law of China. Ovaries of three samples of Dazu black goats were collected from domestic animal conservation field of the Southwest University. The follicles of ovaries were separated into DF (diameter > 5 mm) and nondominant follicles (NF, 3mm < diameter < 5mm), the detail standard protocol, animal description as Supplementary Material.

RNA extraction, library preparation and genome-wide resequencing

Total RNA was extracted using TRIzol® Reagent (Invitrogen, USA). Equal amounts of RNA from three different goats and the same follicle developmental phases (dominant and nondominant) were pooled. Libraries were generated using the rRNA-depleted RNA of the NEBNext

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Abbreviations

DF, dominant follicle; NF, nondominant follicles; RNAseq, transcriptome shotgun sequencing; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; lncRNA, long non-coding RNA; mRNA, messenger RNA; DEGs, differentially expressed genes; FDR, false discovery rate; FRKM, kilobase of exon per million mapped reads; qPCR, quantitative PCR; OXPHOS, mitochondrial oxidative phosphorylation; SDHC, succinate dehydrogenase complex subunit C; Cox11, cytochrome c oxidase assembly protein 11; Cyt1, cystatin; NDUFS1, NADH-ubiquinone oxidoreductase Fe–S protein 1; BMP15, bone morphogenetic protein 15; GDF9, growth differentiation factor 9; IGFBP5, insulin-like growth factor binding protein 5; BMPR2, bone morphogenetic protein receptor type 2; BAX, BCL2 associated X protein; BCL-2, B-cell CLL/lymphoma 2; BMP2, bone morphogenetic protein 2; INHA, inhibiting the alpha subunit of inhibin; PTGS1, prostaglandin-endoperoxide synthase 1; PTGS-2, prostaglandin-endoperoxide synthase 2.

Ultra Directional RNA Library Prep Kit (NEB, Ipswich, MA, USA). Libraries were sequenced on the Illumina HiSeq 2500 platform by Gene Denovo Technologies (Guangzhou, China). The detail libraries optiation and informatic analysis was displyed as Supplementary Material.

Quantitative PCR of candidate RNA factors

The samples used in the q-PCR analyses were the same as those used in the sequencing experiments. The cDNA was synthesized using the First Strand cDNA Synthesis Kit (GE Healthcare) and 1 mg total RNA. The primers are shown in Table I. Quantitative RT-PCR was performed in triplicate using 10 ml SYBR® Select Master Mix (LifeTechnologies), 6.4 μL H_2O, 0.8 ml primer (10 pmol/ml) and 2 μL cDNA (~16 ng). The reaction was performed on an Applied Biosystems StepOnePlusTM Real-Time PCR System (LifeTechnologies). The cycle threshold (Ct) values were normalized to the control gene (*GAPDH*). The relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Each experiment was independently repeated three times, and each sample was evaluated in triplicate. Using the t test, a P value less than 0.05 was considered significant.

RESULTS

Illumina sequencing and gene annotation

Illumina sequencing of *C. hircus* yielded a total of 11401202.75 kb and 14600735 kb raw data from the mixed pools of DF and NF, respectively. The transcriptome sequencing data from the mixed pools of goat follicles were deposited in the NCBI Sequence Read Archive database (accession number, SRA342894). A reference dataset, which included protein data from the goat genome (CHIR_1.0), was constructed for gene annotation. A total of 90276370 kb (DF) and 115579236 kb (NF) clear reads were generated by Illumina paired-end sequencing, and their mapping rate was 84.99% (DF) and 84.47% (NF), respectively. These results demonstrated that both libraries were high quality. Transcripts were blasted against this dataset. Of the total 20763 (DF) and 21821 (NF) transcripts, those with known isoform numbers were 15756 (51.34%, DF) and 16511 (53.80%, NF).

Differential gene expression (DGE) of LncRNA and mRNA between DF and NF

In the analysis of the digital coding gene expression of the follicle tissue in the DF and NF of goats, we found 12,577 significantly differentially expressed genes, including 6,009 genes from the DF that were upregulated compared to the NF and 6,568 that were downregulated.

Differential lncRNA expression in the tissues of the DF and NF from goats, we found 1,026 significant differentially expressed lncRNA, including 419 that were upregulated and 607 that were down regulated.

Functional annotation of the DEGs

A GO analysis was performed to classify the functions of DEGs that had hits in the NCBI NR database. These were summarized under three main GO categories, cellular component, molecular function, and biological process (Fig. 1) and were associated with 56 GO terms in total, which could then be used to implicate the biological processes involved in DF selection.

Table I.- Primer information of candidate gene using q-PCR.

Target	Primer name	Sequences (5'-3')	Ampicon size (bp)
BAX[#]	BAX-F	GCATCCACCAAGAAGCTGAG	130
	BAX-R	CCGCCACTCGGAAAAAGAC	
BCL2[#]	BCL2-F	ATGTGTGTGGAGAGCGTCA	182
	BCL2-R	AGAGACAGCCAGGAGAAATC	
BMPR1B[#]	BMPR1B-F	CCTGTGGTCACTTCTGGATGTC	107
	BMPR1B-R	TTCCGTTCTGTGCAGCATTC	
BMP15[@]	BMP15-F	TGAGGCCGCTGGCTAGTG	147
	BMP15-R	GGGAATGAGTTAGGTGAAGCTGAT	
GDF9[@]	GDF9-F	ACAGACGCCACCTCTACAACACT	136
	GDF9-R	TTCCACAACAGTAACACGATCCA	
IGFBP5[#]	IGFBP5-F	AGAGAGACTCCCGTGAGCAT	159
	IGFBP5-R	ACGAACTTGGACTGGGTCAG	
INHA[#]	INHA-F	CTTCCCTCTGCTGACCCATC	250
	INHA-R	ATTGAGGGCGGCTCTGTG	
BMP2[#]	BMP2-F	A TCACCTGAACTC CACGAA	140
	BMP2-R	TACCACCTTCTCATTCTCATC	
INSIG1[#]	INSIG1-F	GTGGGGAACATAGGACGAC	unknown
	INSIG1-R	ACGAGTCATTTGTACAGTCAGCCC	
BMP6[@]	BMP6-F	CGCCTCAGACTACAACAGCA	165
	BMP6-R	TTCATGTGTGCGTTGAGAGG	
BMPRII[@]	BMPRII-F	AGACCCAAGTTCCCAGAAGC	96
	BMPRII-R	AGCCTCTGCATCCTGGTC	
TGFBR1[@]	TGFBR1-F	CGGAAAGCCGTCATCTGGCCTC	164
	TGFBR1-R	CTCGATGGTGAATGACAGTGCGGT	
PTGS-2[#]	Ptgs2-F	AGGAGGTCTTTGGTCTGGTG	126
	Ptgs2-R	TCTGGAACAACTGCTCATCG	
GAPDH	GAPDH-F	AGGCTGGGGCTCACTTGAAG	223
	GAPDH-R	ATGGCGTGGACAGTGGTCAT	
LOC102183322[#]	LOC102183322-F2	CGGGAGGTTACTGTAGGGC	189
	LOC102183322-R2	TTGCTTAGTTGTCGTCGTGC	
LOC102181730[#]	LOC102181730-F2	ACTGCGAGGACATAACACCA	207
	LOC102181730-R2	CCACTTTCTTCCCCGCTTTC	
LOC102184274[#]	LOC102184274-F2	AGAAGTACAACCCCTCTGCC	230
	LOC102184274-R2	GTCTTCCTTGGGCCTTTTCG	

Note: Gene name with the upper standard. #, identified significant differences in the expression of genes in this study using transcriptome re-sequence; @, the known candidate gene from previous study (Lima *et al.*, 2012; Paradis *et al.*, 2009).
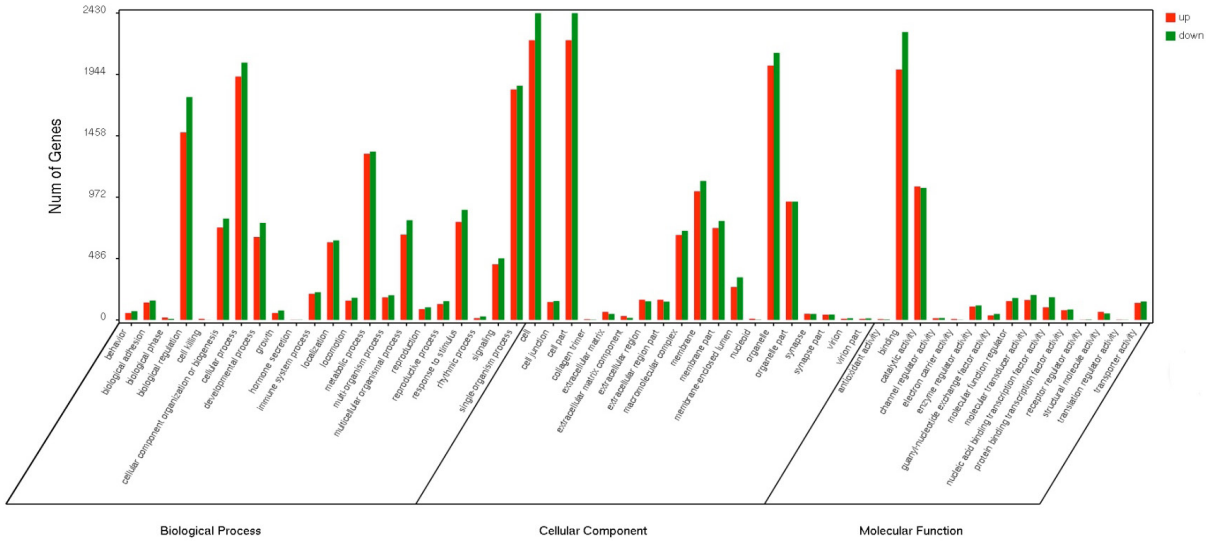
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Fig. 1. Differential expressed gene distribution of gene ontology (GO) categories (level 2) of dominant and indominant follicles mixed pools for goat. GO functional annotations are summarized in three main categories: biological process, cellular componentand molecular function.
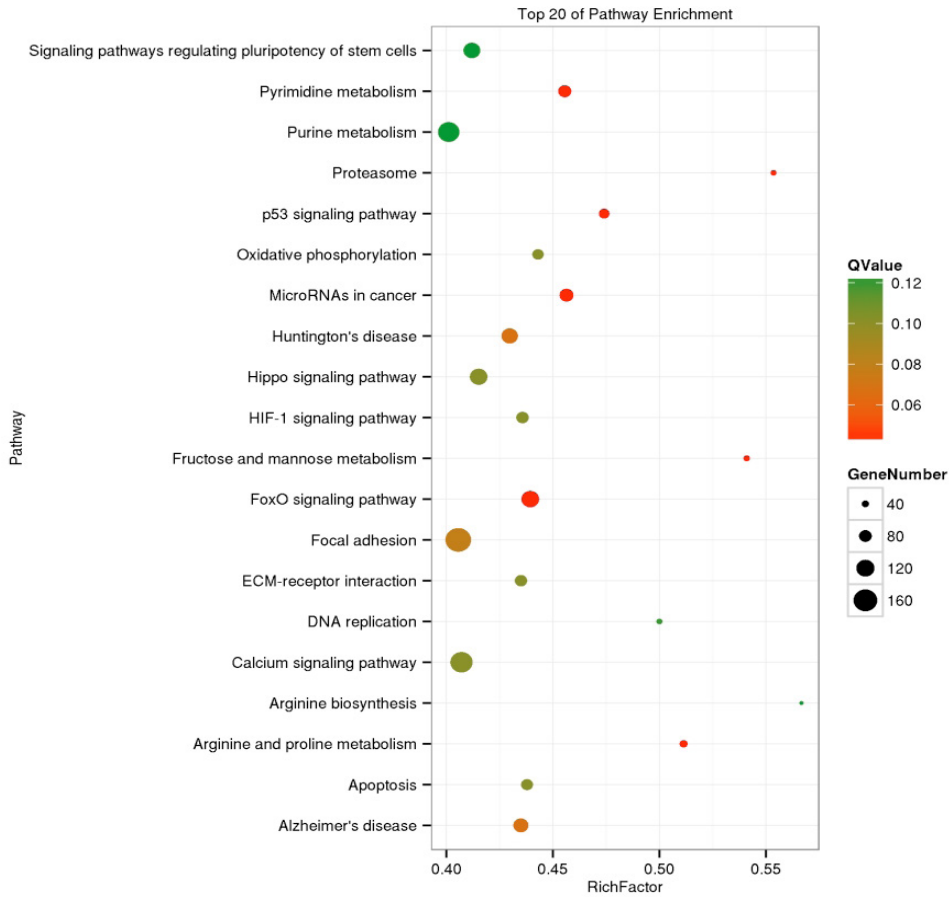

Fig. 2. Top20 enriched pathway of differential expressed coding gene between dominant and indominant follicles in goat.
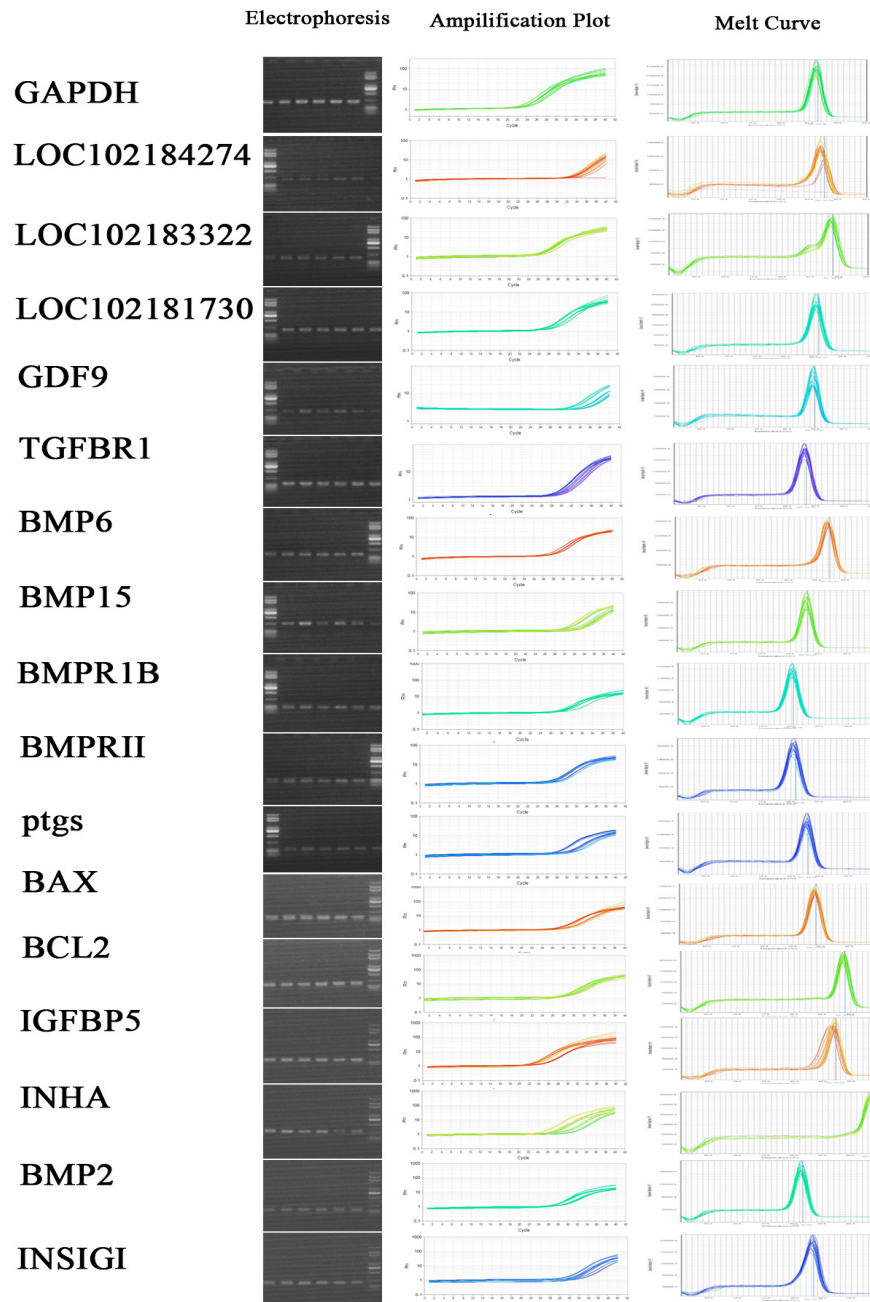
Fig. 3. Candidate transcripts expression pattern of dominant and indominant follicles in goat using q-PCR.

The KEGG database was used to identify potentially important biological pathways in our dataset. A total of 4,318 genes were assigned to 292 KEGG pathways. Furthermore, most of the KEGG pathways involved immune function (*e.g.,* the B-cell receptor signaling pathway, the autoimmune thyroid disease pathway, the Toll like receptor signaling pathway, and reproductive signaling pathways, including the *PPAR* and *GnRH* signaling pathways. Of the 292 KEGG pathways, 41 were enriched (P < 0.05). These annotations provide a valuable resource for investigating specific processes, functions, and pathways in DF selection. In addition, the 20 top pathway enrichments, including signaling pathways regulating the pluripotency of stem cells, the *p53* signaling pathway, and the oxidative phosphorylation pathway, were identified (Fig. 2). Some reproductive related pathways were identified, including oocyte meiosis, ovarian steroidogenesis, progesterone-mediated oocyte

maturation, and the estrogen signaling pathway.

Differential gene expression profiling and qRT-PCR validation

To identify the differentially expressed transcription factors (DETs) associated with DF and NF from the two mixed pools in the current study, we selected some DEGs

for real time quantitative PCR (q-PCR) (Fig. 3). Using this procedure, we found similar gene expression profiles for *INHA*, *IGFBP5*, *BMPR1B*, *BMP2*, *INSIG*, *PTGS2*, *LOC102181730* and *LOC102184274*, which indicated the high credibility of these genes in transcript abundance; however, some factors in the RNA-seq results, including *BAX*, *LOC102183322*, and *BCL2*, were inconsistent (Fig. 4).
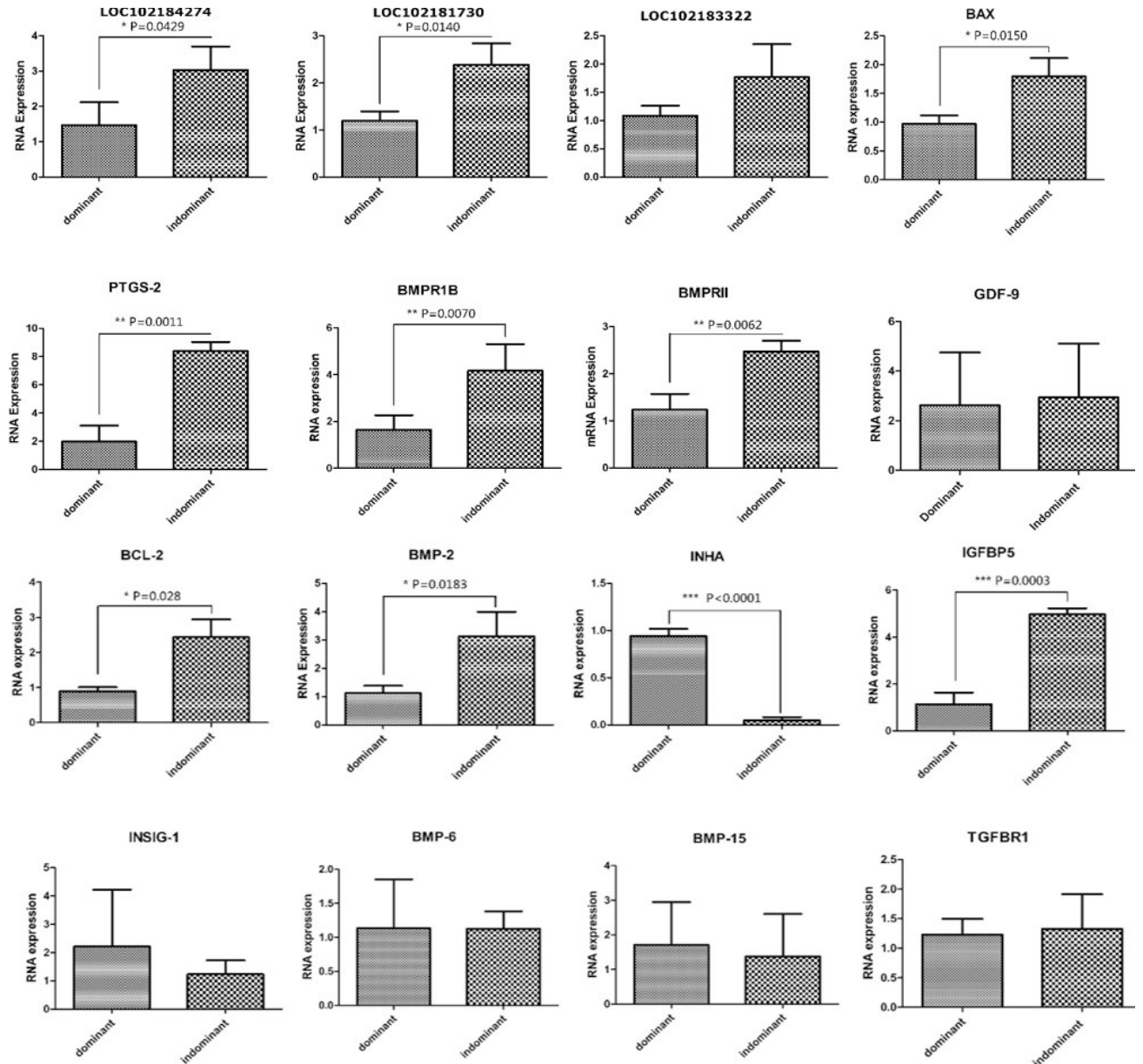

Fig. 4. Real time PCR validation of differentially expressed genes and long nocoding RNA in dominant and indominant follicles. Abundance of target genes was normalized relative to abundance of GAPDH gene. Bars in each panel represent the mean ± standard error (sample number = 3 and 3 parallel repetition per sample), * P < 0.05; ** P < 0.01; *** P < 0.001. BMPR1B, IGFBP5, PTGS-2, BMP2, IGFBP5, BMPR1B, LOC102181730, LOC102184274, LOC102183322 were significant up-regulated expressed in NF in compare with DF; BAX, BCL2, INHA, INSIG were significant up-regulated expressed in DF in compare with NF, which identified from RNA-seq.

DISCUSSION

A total of 1026 lncRNAs (884 novel lncRNAs) and 12577 coding mRNAs with significant differential expression were detected in DF compared to NF. These transcripts were assigned to a total of 56 GO categories. The largest proportion was biological process (22), followed by cellular process (19) and molecular function (15). The results of the present study confirmed that the initial follicle was selected to be the DF by the regulation of various physiological systems

Differentially expressed genes were involved in 292 pathways, of which the 20 most enriched were predicted using KEGG analysis. Including the signaling pathways regulating the pluripotency of stem cells, and research has revealed that the stem cell factor promotes the development of ovarian follicles *in vitro* (Thuwanut *et al.*, 2016). The *p53* signaling pathway, which is a known factor in the regulation of ovarian function, such as the proliferation and the apoptosis of ovarian cells (Sirotkin *et al.*, 2014). The *HIF-1* signaling pathway, has a key role in the development of ovarian tissue, including luteal development (Yu *et al.*, 2015) and ovarian follicular growth (Zhang *et al.*, 2015). In addition, it has indicated that the Hippo signaling pathway has a spatio-temporal correlation with the size of the primordial follicle pool (Xiang *et al.*, 2015).

Ovarian follicles enter a massive growth phase during which they become highly dependent on gonadotrophic factors and nutrients. At the same time, the *FoxO*-dependent process causes prompt and efficient adaptation to nutrient supplies, thereby contributing to maintaining the balance between reproduction and nutrient availability (Jouandin *et al.*, 2014). In addition, there is evidence that indicates that the *FoxO* pathway factor plays a role in follicular development (Li *et al.*, 2014) and in female reproductive disorders (Christian *et al.*, 2011). Thus, the *FoxO* pathway may be important for the regulation of DF selection, too.

However, some pathways were not enriched in this study but may be related to the regulation of DF selection. For example, an interesting pathway, mitochondrial oxidative phosphorylation (*OXPHOS*), takes place inside mitochondria and is highly efficient in releasing energy for biological action. Many studies have found that mitochondrial function is related to reproductive biology (Ben-Meir *et al.*, 2015), particularly the *OXPHOS* pathway is inseparably related to oocyte maturation, fertilization and embryo development (Harvey *et al.*, 2002; Ge *et al.*, 2013). In this study, some genes in the mitochondrial *OXPHOS* pathway, such as succinate dehydrogenase complex subunit C (*SDHC*), cytochrome c

oxidase assembly protein 11 (*Cox11*), and cystatin (*Cyt1*) were differentially expressed.

In addition, the target gene prediction for lncRNAs showed that lncRNA *LOC102181730* and its potential target gene *NDUFS1* were significantly differentially expressed between dominant and non-dominant follicles. In addition, lncRNA *LOC102183322* and its potential target gene *NDUFB6* were also significantly expressed in the DF pool. The *NADH*-ubiquinone oxidoreductase Fe–S protein 1 (*NDUFS1*) and the *NADH*-ubiquinone oxidoreductase subunit B6 (*NDUFB6*) belong to *NADH* dehydrogenase (ubiquinone) is related to oxidative-phosphorylation (*OXPHOS*). Some studies have found that a variant in *NDUFS1* resulted in a complex I deficiency (Björkman *et al.*, 2015). *NDUFB6* is required for complex I activity, and it defines conditions suitable for a systematic and stable exclusion of the different supernumerary subunits in human cells (Loublier *et al.*, 2011). These results indicate that the interaction of *LOC102183322* and *LOC102181730* with *NDUFS1* and *NDUFB6* might regulate *OXPHOS*. Here, we believe that this biological process plays a role in DF selection.

Some candidate genes were identified from previous study were controversy, including growth differentiation factor 9 (*GDF9*) promotes growth of the oocyte at the primary (Cook-Andersen *et al.*, 2016; Kona *et al.*, 2016; Pramod *et al.*, 2013). In addition, numerous studies have revealed that bone morphogenetic protein 15 (*BMP15*) is differentially expressed in small and large antral follicles during the development *in vitro* of cultured preantral follicles (Pramod *et al.*, 2013; Lima *et al.*, 2012). However, some reported these genes (*BMP15* or *GDF9*) to be inactive in reproductive functions because no differences were identified that were associated with enhanced ovulation rate (Feary *et al.*, 2007), it was consistent with this study.

In previous study, it has been shown that the bone morphogenetic protein receptor type 2 (*BMPR2*), the insulin-like growth factor binding protein 5 (*IGFBP5*), the *BCL2* associated X protein (*BAX*) and the B-cell CLL/lymphoma 2 (*BCL-2*) protein have different transcription patterns in different stages of follicles cultured *in vitro* (Lima *et al.*, 2012; Yang *et al.*, 2012; DeBem *et al.*, 2014). These patterns are consistent with the expression patterns observed in the current study. However, the *BMP6*, *BMP15* and *GDF9* mRNAs were most abundant in the oocyte, and their expression remained relatively constant during follicular development, whereas the *BMPR1B* and *TGFBR1* were temporally regulated in different stages in pigs (Paradis *et al.*, 2009). However, the expression of *GDF9*, *BMP15*, *BMP6*, and *TGFBR1* exhibited some differential expression in our study, and *BMPR1B* showed significantly different expression in DF and NF. It indicated

that the regulated transcription in the selection of DF was species-specific.

Bone morphogenetic protein 2 (*BMP2*), which showed significantly different expression in DF and NF in this study, has been shown to regulate primordial follicle formation by promoting the germ cell to oocyte transition and the somatic cell to pre-granulosa cell formation (Chakraborty and Roy , 2015). Inhibiting the alpha subunit of inhibin (*INHA*), which is related to infertility (Rah *et al*., 2014) and ovarian insufficiency (Li *et al*., 2015). Prostaglandin-endoperoxide synthase 2 (*PTGS-2*) and prostaglandin-endoperoxide synthase 1 (*PTGS1*), also known as the cyclooxygenases, are key enzymes in prostaglandin biosynthesis and act both as dioxygenases and as peroxidases. Both genes showed significant differential expression in this study. Therefore, *BMP2*, *INHA* and *PTGS-2* would be novel candidate genes for DF selection in goats.

The results of q-PCR that were not complete consistent with the results of RNA-seq (Consistency rate = 72.72%) indicated that this study design was not optimal. The lack of replication was due to using two mixed pooled samples for the transcriptome sequencing analysis, resulting in a certain number of false positives. Despite such limitations, our results enhance the understanding of the regulation of the selection of the DF and lay the foundation for future studies.

CONCLUSION

In this study, the lncRNA and mRNA profiles of DF selection in goats were investigated using RNAseq. A total of 1026 lncRNAs and 12577 coding RNAs were detected that were differentially expressed in the two mixed pools of follicles. Further functional annotation analysis indicated that some significant biological categories and signaling pathways were involved in DF selection. In addition, some candidate RNAs that provide a new perspective on the mechanisms regulating this physiological process were identified by q-PCR. In short, the results of the current study provide a valuable basis for understanding the molecular mechanisms of DF selection in goats.

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