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Exploring differentially expressed genes in the ovaries of estrous and anestrous Qira black sheep using RNA-seq technique

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ABSTRACT

Ovary is the main functional organ in reproduction and has obvious difference in the biological activity during the estrous cycle. To investigate the genes associated with follicular development, ovulation, or non-season reproduction, gene expression differences in the ovaries of Qira black sheep estrous stage and anestrous stage in May were assessed using RNA-seq technology. This analysis obtained approximately 1.17 Gb and 1.22 Gb clean reads in Qira black sheep anestrous stage and estrous stage libraries, respectively. Six hundred and fifty one differentially expressed genes (DEGs) were identified, in which 400 genes were up-regulated and 251 genes were down-regulated in the anestrous stage samples compared with estrous stage samples. GO enrichment analysis revealed that these DEGs were significantly enriched in 52 functional groups for the 3 GO categories. Using KEGG pathway analysis, 35 significant signaling pathways were enriched, including calcium signaling pathway, steroid biosynthesis, cell adhesion molecules, and steroid hormone biosynthesis. This study will provide a list of candidate genes for the future research in sheep reproduction.

Key words: Anestrous, Differentially expressed genes, Estrous, Ovary, Qira black sheep

As a high throughput sequencing technology, RNA-seq provides a platform for measuring large-scale gene expression patterns (Zhao *et al*. 2015). This platform is commonly used to survey the transcriptome and genome-wide differences in gene expression between samples (Chen *et al.* 2015). RNA-seq is now widely used to identify the DEGs and novel transcript units in mammalian reproductive tissues, for example in the ovaries of uniparous and multiparous goats (Ling *et al.* 2014), bovine granulosa cells (Hatzirodos *et al.* 2014), bovine blastocysts (Chitwood *et al.* 2013), sheep granulose cells and oocytes (Bonnet *et al.* 2011), and sheep ovaries (Miao and Luo 2013, Chen *et al.* 2015). So far, studies on the sheep reproductive related genes that have applied RNA-seq technology are scarce. Only Miao and Luo (2013) and Chen *et al*. (2015) investigated the gene expression in ovaries of high-fecundity sheep and low-fecundity sheep using RNA-seq technology to identify the possible genes related to sheep prolificacy.

Sheep, though an important domestic animal in China yet majority of breeds are seasonal breeders and produce single lamb, greatly affecting the sheep fecundity and development of sheep industry. Ovary, the main functional organ in reproduction, performs numerous roles critical for oocyte development and ovulation. Obvious differences were found in the biology, activity and endocrine characteristics of the ovary during the estrous cycle (Bartlewski *et al.* 2011). Chen *et al.* (2015) in studies on gene expressions using ovaries during the estrous cycle, concentrated on investigating only a select few genes and not on a global scale.

In Northwest China, the Qira black sheep is a famous local breed because of its year-around estrous behavior, high-fecundity with an average lambing rate of 215.5% and lambskin. In this study, the expression profiles of the mRNAs in Qira black sheep ovaries were compared between anestrous and estrous stage in May (non-season) using RNA-seq technology. This discovery may provide a list of candidate genes for future research of sheep reproduction, such as follicular development, ovulation and the maintenance of estrus.

MATERIALS AND METHODS

Animal and ovary collection: Qira black ewes with similar age (3–4 years old) and appearance body size were were housed in 1 group under the normal condition of natural lighting and free access to feed. Four Qira black ewes were slaughtered humanely by anesthesia after spontaneous estrus in May was detected. At the same time, 4 Qira black ewes were slaughtered in anestrous stage. All

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ovary samples were immediately frozen on liquid nitrogen for total RNA extraction.

RNA isolation and RNA-seq: According to the manufacturer's instructions, total RNA was extracted from ovaries in the 2 experimental groups using Trizol reagent, and then any possible DNA contamination was degraded using DNase I. The sequencing libraries were performed and used for sequencing on an Illumina HiSeqTM 2000 instrument at Beijing Genomics Institute.

Bioinformatics analysis: The raw reads were filtered to remove the low quality, contaminated sequences, and sequencing adapters. The clean reads were aligned to the sheep reference gene sequences (downloaded from NCBI database) and sheep genome (oar3.1) through SOAP2 (Li *et al.* 2009). Unmapped or multi-position matched reads were excluded from further analyses.

Expression profiling: The RPKM (reads per kb per million reads) method was used to calculate the expression level of each gene. Statistical significance of differential expression of each gene was determined (Audic and Claverie 1997, Wang *et al.* 2010).

Gene ontology (GO) and KEGG pathway enrichment analysis: The hypergeometric test was used to map all DEGs in GO terms (http://www.geneontology.org/) and identify the significantly enriched GO terms comparing to the genome back (Ashburner *et al.* 2000). WEGO software was used to perform the GO annotation analysis and plotting GO annotations (Ye *et al.* 2006). Meanwhile, the biological pathway annotations were identified using the KEGG database (Kanehisa *et al.* 2008).

RESULTS AND DISCUSSION

Sequencing data summary: Construction and sequencing of the ovary transcriptome library of Qira black sheep in estrous period were summarized earlier (Chen *et al.* 2015). In this study, approximately 2.42 Gb raw reads (1.19 and 1.23 Gb for Qira black sheep in estrous and anestrous period respectively) were obtained. Next, according to the BGI bioinformatics protocols for RNA-seq, these data were preliminarily analyzed. After removing the low quality, contaminated sequences and sequencing adapters, more than 1.17 Gb clean reads in each library were obtained (Table 1), and the percentages of clean reads among the raw reads reached 98.61% and 98.94% in the Qira black sheep estrous and anestrous ovary libraries, respectively. The rates of total mapped reads were more than 82% for Qira black sheep in estrous and anestrous ovary libraries (Table 1). For the unique match, a little more than two-thirds of the reads corresponded to reference genome.

Identification and analysis of DEGs: The RPKM method was used to evaluate the gene expression levels. As a result, 17,166 genes were detected, with 397 genes expressed only in estrous stage and 403 genes expressed only in anestrous stage. A total of 651 DEGs were identified between the two libraries, in which 400 genes were up-regulated and 251 genes were down-regulated in the Qira black sheep anestrous stage library compared with estrous stage library (Table 1; Fig. 1). Some of the DEGs have been previously associated with reproduction processes, such as CYP17A1 (steroid 17-alpha-hydroxylase/17, 20 lyase), PGR (progesterone receptor), LSHR (Luteinizing hormone receptor), LHß (lutropin subunit beta), CYP11A1 (cholesterol side-chain cleavage enzyme), HSD3B1 (3 beta-hydroxysteroid dehydrogenase/Delta 5—>4-isomerase type 1), PRLR (prolactin receptor), VEGFR2 (vascular endothelial growth factor receptor 2), and VLDLR (low-density lipoprotein receptor isoform 2). While other genes may be involved in the follicular development, control of ovulation or secretion of steroid hormone. For example, we identified the INSL3 (insulin-like 3 precursor) up-regulated, and STAR (steroidogenic acute regulatory protein), HIF1ß (hypoxia-inducible factor 1-alpha isoform 3) down-regulated in Qira black sheep estrous stage library. INSL3 is a member of the relaxin-insulin family. Previous work had suggested that INSL3 might be an important intrafollicular modulator of theca interna cell function/steroidogenesis (Satchell *et al.* 2013), and theca cell-derived growth factor for preantral follicle (Xue *et al.* 2014). STAR

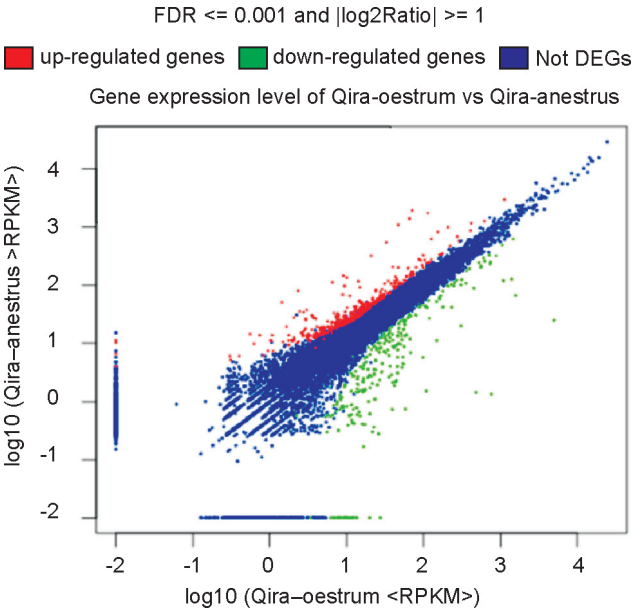

Fig. 1. Comparison of gene expression levels between the two libraries. The expression levels are estimated by RPKM value.

Table 1. Read numbers and mapping results for the two constructed Qira black sheep ovary libraries

Sample ID	Total reads	Total mapped reads	Unique match	Multi-position match	Total unmapped reads
Estrous stage	11747582(100.00%)	9679005(82.39%)	8052464(68.55%)	1626541(13.85%)	2068577(17.61%)
Anestrous stage	12217336(100.00%)	10230743(83.74%)	8526101(69.79%)	1704642(13.95%)	1986593(16.26%)

is essential for steroid hormone synthesis and involved in the regulation of follicular development in the mammalian ovary (Adib *et al.* 2014). Another example is HIF1ß, which is a subunit of HIF1 (hypoxia-inducible factor 1). HIF1 has been implicated to be a critical mediator of the processes of follicle development and ovulation and regulator of ovarian gene expression (Rico *et al.* 2014, Navanukraw *et al.* 2014). In summary, all of these results suggested that plenty of the DEGs are potentially critical for regulating sheep fecundity.

Interestingly, the fold change of the DEGs identified in the present study ranged from 11.43– to 1.0-fold, and the number of DEGs more than 2–fold difference accounted for 35.64% of total DEGs (Table 2). Some of the DEGs with high fold change are the important candidate genes for future analysis. For example, the prolactin-releasing peptide (encoded by PrRP or PRLH) promotes the release of prolactin from the pituitary, and prolactin plays a significant impact on the animal reproduction and (Thompson and Oberhaus 2015). Oviduct-specific glycoprotein (encoded by OVGP1) promotes the release of oviductin under the control of ovarian steroids and specifically expressed during postovulatory phase of the estrous in oviduct, and this protein was implicated in the regulation of fertilization and early embryonic development (Saint-Dizier *et al*. 2014, Yang *et al.* 2015). Moreover, some of DEGs with relatively small fold change might also have large effects on reproduction processes involved in follicular development, ovulation, or others (Garosi *et al.* 2005). Therefore, further studies are required to investigate the functional characterization of these genes.

Table 2. Fold change differences in expression of genes differentially expressed between the two libraries at FDR < 0.001

	Fold change		
	> 4	2–4	dþ2
Genes over-expressed in anestrous stage	16	65	319
Genes over-expressed in estrous stage	57	94	100

GO and pathway enrichment analysis: GO terms are classified into 3 categories: biological process, cellular component, and molecular function. In this study, the 651 DGEs could be categorized into 52 functional groups, in which 23, 17, 12 functional groups were identified for the 3 GO categories (Fig. 2). The GO annotation indicated that the DEGs were involved in many biological processes, such as the cellular process, single-organism process, and reproduction. The predominant functional groups in cellular component are cell and cell part, organelle, and membrane, and in molecular function are catalytic activity and binding.

The results of KEGG pathway significant enrichment analysis showed that 283 DEGs had KEGG pathway annotations and participated in 168 pathways. In total, 35 significant signaling pathways were enriched, including

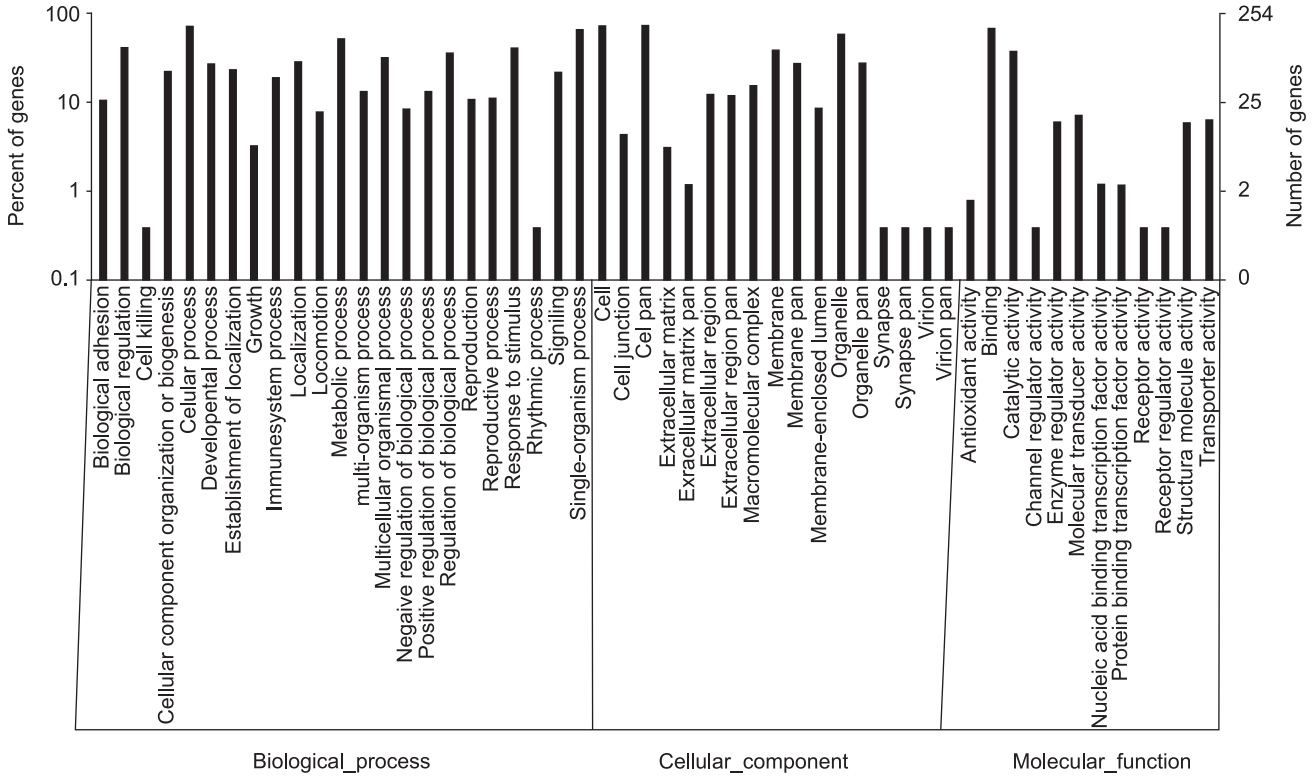

Fig. 2. GO analysis of differentially expressed genes between the two libraries. The differentially expressed genes are classified into three categories: cellular component, molecular function, and biological process. The left axis shows the percentage of genes in a category, and the right axis the number of genes.

calcium signaling pathway, cell adhesion molecules, steroid biosynthesis, and steroid hormone biosynthesis. Most of the hormone related genes identified in this study involved in steroid biosynthesis and steroid hormone biosynthesis pathways up-regulated Qira black sheep anestrous stage. For steroid hormone biosynthesis pathway, four of five genes (CYP11A1, HSD3B1, and others) were up-regulated and one gene (CYP17A1) down-regulated in Qira black sheep anestrous stage. Moreover, VLDLR and STAR were also up-regulated in Qira black sheep anestrous stage, and involved in steroid hormone biosynthesis through effects on the absorption of cholesterol substrates and the transportation of intracellular cholesterol respectively (Ying *et al*. 2013). Previous studies have indicated that calcium signaling pathway plays critical roles in steroidogenesis and mammalian oocyte maturation (Abdou *et al*. 2013). Cell adhesion molecules (CAMs) are cell surface proteins involved in many biological processes such as cell adhesion, proliferation, differentiation, and the developmental processes of fertilization, embryogenesis, and embryonic development (Sun and Xie 2012, Simopoulou *et al*. 2014). In the present study, most of the DEGs involved in calcium signaling pathway (20/22) and CAMs (14/17) were up-regulated in Qira black sheep estrous stage. These results indicate that these DGEs involved in calcium signaling pathway and CAMs pathway may promote the follicular development, ovulation. Furthermore, some molecules of other pathways involved in the reproduction processes are also differentially expressed, such as gonadotropin-releasing hormone 2 receptor and mitogen-activated protein kinase 13 in GnRH pathway, and c-Fos and CD14 in MAPK pathway.

In addition, plenty of genes involved in cell cycle (such as S-phase kinase-associated protein 1 and protein FAM81A), linked to mitochondrial (kinase isozyme 3, 28S ribosomal protein S35), and proteins required for transport (such as choline transporter-like protein 4 isoform 1, and zinc transporter 3) were also differentially expressed. Nonetheless, further studies are required to identify the biochemical and physiological function of some critical DEGs, such as the exact functions in follicular development, ovulation and regulatory networks.

Present study concluded that 651 DEGs were obtained in the ovarian tissues of the Qira black sheep estrous stage and anestrous stage in May. GO and pathway analysis indicated that 52 functional groups and 35 significant signaling pathways were identified. The results indicated that plenty of the DEGs identified in this study might play important roles in sheep reproduction processes.

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