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Transcriptome studies of granulosa cells at different stages of ovarian follicular development in buffalo

J. Li[a,b], Z. Li[a,b], S. Liu[a,b], R. Zia[a], A. Liang[a,b], L. Yang[a,b,*]

[a] College of Animal Science and Technology, Huazhong Agricultural University, Wuhan, Hubei, China
[b] Hubei Province's Engineering Research Center in Buffalo Breeding & Products, China

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ABSTRACT

The normal maturation and ovulation from ovarian follicles is important in ensuring conception and improving fertility of buffalo. The molecular regulation mechanism of buffalo follicles growth, however, remains unknown. This study analyzed the gene expression profiles associated with buffalo ovarian follicle growth. According to the analysis of RNA sequencing, 17,700 unigenes and 13,672 differentially expressed genes (DEGs) were detected. A total of 30 common DEGs were identified during four stages of follicle growth, and the expression patterns are basically synchronized, suggesting the products as a result of expressions of these genes may cooperate to regulate follicular development. Furthermore, GO and KEGG enrichment analyses revealed that the majority of DEGs in early stage of follicular growth were enriched in ribosomal and oxidative phosphorylation signaling pathways, and the expression patterns of these DEGs are basically up-regulated at the beginning of follicular growth (< 8 mm, diameter), and then down-regulated (8–12 mm) in the following stages of follicular development. The pathway of immune signaling, including allograft rejection, chemokine signaling pathway, natural killer cell mediated cytotoxicity, phagosome, and antigen processing and presentation, was significantly enriched in the last stage of follicular development (> 12 mm), which indicates that the immune system has an important role in the last stage of follicular maturation and ovulation. This study provided a gene expression profile of buffalo follicle growth, and provided an insight into biological processes associated with molecular regulation of ovarian follicle growth.

1. Introduction

Buffalo, one of the most important economic livestock worldwide, is known for its resistance to stress and its milk product with high milk fat and protein contents. The number of buffalo stock worldwide is estimated to be approximately 172 million (FAO: http://faostat.fao.org/). Unlike swine, sheep, and goats, however, where multiple follicles undergo maturation and from which ovulation occurs, buffalo is a monovular species, and thus, it normally has only one follicle develop into mature follicle from which ovulation occurs (Ginther et al., 2001) resulting in a lesser fecundity in buffalo. This is an important factor restricting the development of the buffalo breeding industry.

Ovaries are critical organs for follicular development and ovulation. The follicle is the structural and functional units of the ovaries for which normal development during the developmental stages is important for lifelong fertility of buffalo. Follicle development results with granulosa cell growth, development, differentiation, and apoptosis in follicles, which is closely related to growth of oocyte and follicular atresia (Jain et al., 2016; Yeung et al., 2017). Two or three waves of ovarian follicular development emerge

* Corresponding author at: No.1 Shizishan Street, Wuhan, Hubei Province, 430070, China.
E-mail address: liguoyang2006@163.com (L. Yang).

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Table 1
Results of RNA-seq, assembly, and annotion.

Stage	Raw reads	Clean reads	Assembled reads	Unigenes	DEGs	SDEGs
GC1	50,760,648	46,219,792	29,117,053	16,249	–	–
GC2	50,760,170	46,675,486	29,120,413	16,339	13160	299
GC3	50,760,644	46,192,560	30,062,845	16,167	13048	303
GC4	54,035,416	46,066,398	28,098,004	16,349	13228	691
Total	206,316,878	185,154,236(89.74%)	116,398,315(62.87%)	17,700	13,672(77.24%)	1076(7.87%)

DEG, differentially expressed genes based on the previous stage; SDEG, significantly differentially expressed genes based on the previous stage with FDR ≤ 0.05 and $|\log_2 Ratio| \geq 1$.

during each estrous cycle (Fortune et al., 1991), and the emergence of each follicular wave is synchronous with the surge of a follicle-stimulating hormone (FSH) (Evans et al., 1997). The FSH has a pivotal role during the early stage of follicular development, ensuring follicle recruitment and growth of these structures (Adams et al., 1992) until the dominant follicle reaches a certain size (i.e., 7.2 mm in buffalo) (Gimenes et al., 2011). Furthermore, a fertilizable oocyte is extruded from the follicle as a result of the pre-ovulatory surge of gonadotropins. Knowledge about follicular substances and the effects on follicle growth, however, as well as the diameter deviation mechanism of the largest from subordinate follicles during a wave of ovarian follicular development is limited.

The roles of several key regulator genes involved in follicle growth have been identified (Evans et al., 2004; Tsuiko et al., 2016), however, the mechanism of the underlying global regulatory networks at the transcriptome level is still poorly understood. Transcriptome assembly knowledge has been applied to exploring transcriptional regulation mechanisms of many species, such as dairy cattle (Salleh et al., 2017), sheep (Jäger et al., 2011), and pigs (Du et al., 2014). In the present study, four independent cDNA libraries were constructed, respectively, representing four buffalo follicle growth stages by Illumina RNA-seq. The transcriptome changes during follicle growth were analyzed so as to reveal the molecular mechanism of follicular development. Furthermore, this is the initial attempt to report the dynamic development of buffalo follicles at transcriptome level.

2. Materials and methods

2.1. Material and sample collection

In the present study, ovaries were collected at local abattoir in Guangxi, China, from non-pregnant hybrid buffalo. The ovaries with luteinized and large cystic follicles were detected by macroscopical examination and were not used in the study. Only the one largest follicle from each ovary was collected. The morphological characteristics were used to assess the developmental stages of follicles. Size is the most obvious morphological characteristics. In this study, size of follicles were designated into four categories: GC1 (< 5 mm in diameter), GC2 (5–8 mm), GC3 (8–12 mm) and GC4 (> 12 mm) with number in each category being 44 (Ginther et al., 2003; Pandey et al., 2011). Granulosa cells and follicular fluid were collected (Hatzirodos et al., 2014) from all follicles. Mixed granulosa cell samples representing each stage (n = 44) were frozen immediately in liquid nitrogen, and stored at −80 °C until RNA-seq and RT-PCR. The follicular fluid samples of each stage were collected with three replicate samples for hormone measurement.

2.2. RNA extraction, cDNA synthesis and sequencing

Total RNA was extracted from the granulosa cells of all follicles by using E.Z.N.A total RNA Kit I (R6834-02) according to the manufacturer's instructions, and RNase-free DNase (Takara, China) was used to remove genomic DNA contamination. A total of 4 μg purified RNA per sample was sent to Hanzhou One Gene Co., where the samples were used to construct cDNA library. The library was constructed by using the NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA) according to the manufacturer's instructions. In addition, the Agilent Bioanalyzer 2100 system was used for assessing the quality of library. The, Illumina HiSeq™ 4000 was subsequently used for sequencing the amplified fragments and 150-bp paired-end reads were obtained by Hanzhou One Gene Co. (Hanzhou, China).

2.3. Assembly and annotation

It is necessary to perform quality control on raw data, including removal of the raw data containing adapter, low quality reads with quality value Q < 20 base greater than 30%, and more than 5% unknown nucleotides. The software SOAPaligner/SOAP2 (Li et al., 2009) was used to compare the "clean" data of each sample with the reference gene of the species, allowing up to five base mismatches. The RPKM was used to obtain the relative gene expression amounts (Mortazavi et al., 2008). The analysis of the differentially expressed genes was performed by using the edgeR package in Bioconductor. The DEGs were screened among comparison groups according to the principle of FDR ≤ 0.05 and | log2Ratio | ≥1 so as to control the false discovery rate (Benjamini and Yekutieli 2001). All the DEGs were combined to perform K-means clustering, and the genes were grouped according to various amounts of expression. Venn diagrams and clustering heat maps in the present study were generated using Venn diagram and Pheatmap packages in R based on the extent of gene expression of DEGs for each comparison. Unigenes were compared to the NR library using the blast software, while GO annotation information for all genes was extracted from the Gene Ontology database (http://www.geneontology.org/). Then, GO and KEGG pathway analyses were performed (Moriya et al., 2007; Young et al., 2010).