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Transcriptome reveals B lymphocyte apoptosis in duck embryonic bursa of Fabricius mediated by mitochondrial and Fas signaling pathways

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ABSTRACT

As a central immune organ unique to birds, the bursa of Fabricius (BF) provides a proper microenvironment for B-cell development. The bursal B-cells undergo rapid proliferation and differentiation at the embryonic stages, but 95% of them undergo apoptosis after hatching. Few studies have focused on the cause of bursal B-cells apoptosis at the embryonic stages in birds. To explore the cause, we compared the transcriptional profiles of three characteristic embryonic stages in duck, including embryonic day 14 (ED14), 22 (ED22) and 1 day after hatching (D1). Our results showed that the apoptotic B-cells were first observed at ED22 while there were no apoptotic B-cells at ED14. By performing enrichment analysis for DEGs and qRT-PCR, our results demonstrated that both mitochondrial and Fas signaling pathways mediated bursal B-cell apoptosis during the duck embryonic development. Further, protein-protein interactions (PPIs) and KEGG enrichment analysis together showed that BMP4, FoxO1 and IGF-1 may regulate bursal B-cells apoptosis. In addition, the DEGs showed two stage-specific expression patterns. By analyzing the genes of two expression patterns, the results indicated that B-cell false differentiation may be one of the reasons of apoptosis in the duck embryonic BF. Overall, these data demonstrated that from ED14-ED22, apoptosis of bursal B-cells was mediated by mitochondrial and Fas signaling pathways and could be regulated by BMP4, FoxO1 and IGF-1 in duck. One of the primary causes of bursal B-cell apoptosis may be false differentiation in B-cells.

1. Introduction

The bursa of Fabricius (BF) is a primary humoral immune organ that is evolutionarily unique to birds (Davuson, 2008). The mature bursa contains more than 10,000 follicles that provide an appropriate microenvironment for B-cell development (Mustonen et al., 2010). In BF, B-cells can produce various kinds of antibodies after undergoing immunoglobulin gene diversification (Glick et al., 1956; Ratcliffe, 2006). The secreted antibodies have the ability to bind to pathogens and mediate their elimination via the humoral immune response. In addition, the bursa can also secrete some biologically active factors, including Bursin, BP5, and BASP. These factors can enhance the immune response and induce lymphocyte development (Lassila et al., 1989; Li et al., 2011; Moore et al., 2003).

The development of chicken BF can be divided into four phases. BF primordium can be detected on the 4th day of the chicken embryonic stage in the cloacal region (Douarin and Belo, 1975). During the 8-11th embryonic days, the stem cells enter the BF and develop into prebursal B-cell progenitors in BF (Glick, 1995). During the 11–14th embryonic days, lymphoid follicles of BF start to form, and B-cells undergo rapid proliferation processes in the follicles (Nagy and Oláh, 2010). Around the time of hatching, most B-cells undergo apoptosis in bursal follicles (Mustonen et al., 2010). Only 1%–5% of the B-cells produced per day can migrate to the periphery (Lassila, 1989). Finally, at the stage of avian sexual maturity, BF will develop to involute (Thompson et al., 1986).

With the development of BF, B-cells undergo immigration, proliferation, differentiation and emigration. However, 95% of bursal B-cells produced per day die due to apoptosis (Lassila, 1989). B-cell apoptosis is prominent in bursal follicles after hatching (Neiman et al., 1994). During chicken embryo development, the apoptotic cells are distributed in the mesenchymal cells, the caudal cloacal epithelium, and the epithelial bud (Makino et al., 2014). In post-hatching ducks, apoptotic bursal cells were mainly found on the follicular cortex and

Abbreviations: BF, bursa of Fabricius; ED14, embryonic day 14; ED22, embryonic day 22; D1, day 1 after hatching; DEGs, different expressed genes; PPI, protein-protein interaction; KEGG, Kyoto encyclopedia of genes and genomes; GO, Gene ontology

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medulla (Fang, 2014). In mammals, previous studies showed that death-by-neglect and death-by-instruction were the major signaling pathways regulating B-cell apoptosis in the germinal center (GC), which plays an essential role in maintaining mammalian homeostasis (Rathmell and Thompson, 2002). However, the detailed signaling pathways that mediate B-cells apoptosis have rarely been investigated in avian BF. Previous studies proved that only some genes, including Bcl-2, Bax, Fas, FasL, Nr13, and growth hormone, were associated with bursal B-cells apoptosis (Fang et al., 2011; José Luis et al., 2015; Lee et al., 1999). These studies referred to bursal B-cell apoptosis being more concentrated in the stages of post-hatching poultry.

The embryonic phase is an important period, and it plays a crucial role in organ formation, development, and physiological functions. Studies showed that bursal B-cells undergo rapid proliferation in the embryonic bursal follicles (Nagy and Oláh, 2010). Further, a gene conversion occurred in the embryonic follicles that led to pre-immune immunoglobulin gene diversification (Mccormack and Thompson, 1990; Reynaud et al., 1987). Prior to the experiments described here, we developed a double immunofluorescence staining method. Using this staining method, we found proliferating and apoptotic B-cells in the BF. These were present in bursal follicles at ED22 and D1. The objective of the present study was to investigate global gene expression in the duck BF during the embryonic period. Comparisons were made at the transcriptome level to explore the mechanisms of B-cell apoptosis and homeostatic regulation. The current study may provide a model for studying abnormal B cell apoptosis and peripheral B cell death, which may provide a reference for studies on B cell-related disease therapy.

2. Materials and methods

2.1. Sample collection

Fertilized duck eggs (Nonghua-JS strain) were provided by Sichuan Agricultural University Waterfowl Breeding Experimental Farm (Ya'an, China). The eggs were hatched and maintained under identical conditions. The BF samples were collected from duck embryos at ED14 and ED22 and from ducklings at D1. For each stage, three replicating sample pools were prepared. Each sample pool contained three individual organs. The ED14 sample pools were named T01, T02 and T03. The ED22 samples were named T04, T05 and T06. The D1 samples were named T07, T08 and T09. Samples were immediately frozen in liquid nitrogen and stored at −80 °C before RNA isolation. Other parts of samples for each stage were stored in paraformaldehyde. All animal experiments were authorized by the Animal Ethics Committee of Sichuan Agricultural University.

2.2. Histologic staining and double immunofluorescence staining

The samples fixed in 4% buffered paraformaldehyde were dehydrated with an ethanol series, embedded in paraplast and cut into 5 μm sections, as described by Fang (Fang, 2014). Some sections were stained with hematoxylin and eosin (Servicebio, Wuhan, China), dehydrated, and covered with neutral balsam. Others sections were used for double immunofluorescence staining. The Image-Pro-Plus 6.0 software (Media Cybernetics, MD, USA) was used for microscope analysis.

CD79a was used as a B-cell marker for immunophenotyping (Chu and Arber, 2001) and PCNA was used to test proliferating cells (Addison et al., 2002). The double immunofluorescence staining assay was performed as described by Olson (Olson et al., 2006). TUNEL assay Kit (Roche, Basel, Switzerland) was used according to the manufacturer's instructions. Monoclonal antibodies against mouse CD79a (Abcam, Cambridge, UK) were diluted 1:100, and antibodies against rabbit PCNA (Servicebio, Wuhan, China) were diluted 1:100. Goat anti-mouse antibodies were diluted 1:300 (Servicebio, Wuhan, China), and goat anti-rabbit antibodies (Servicebio, Wuhan, China) were diluted 1:400. The images were captured using an inverted fluorescence microscope (Nikon, Tokyo, Japan), and a computer equipped with an imaging system (Nikon, Tokyo, Japan).

2.3. RNA extraction, cDNA library construction and Illumina sequencing

Total RNA of nine samples was extracted separately using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were measured using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system System (Agilent Technologies, CA, USA). RNA samples that passed the quality tests were used for subsequent sequencing analysis. High-quality RNAs were sent to Biomarker Technologies Corporation (Beijing, China) for cDNA library construction and sequencing. The mRNA was purified by the interaction of the poly (A) tails and magnetic oligo (dT) beads. RNA sequencing libraries were generated using the NEBNext® Ultra RNA Library Prep Kit for Illumina (New England Biolabs, MA, USA) with multiplexing primers according to the manufacturer's protocol. The cDNA library was constructed with average inserts of 200 bp (150–250 bp) and with the non-stranded library preparation. The cDNA was purified using AMPure XP Beads (Beckman Coulter, CA, USA). The short cDNA fragments were subjected to end repair and adapter ligation. The suitable fragments were selected using Agencourt AMPure XP beads (Beckman Coulter, CA, USA) and enriched by PCR amplification. Sequencing was performed via a paired-end 150-cycle rapid run on the Illumina HiSeq.

2.4. Data filtering and de novo assembly

High-quality clean reads were obtained by removing the adaptor sequences, duplicated sequences, ambiguous reads ('N'), and low-quality reads. Transcriptomes were separately assembled *de novo* using Trinity (http://trinityrnaseq.sourceforge.net/). Clean reads with a certain overlap length were initially combined to form long fragments without N (named as contigs). Related contigs were clustered using the TGICL software to yield unigenes (without N) that cannot be extended on either end. Redundancies were removed to acquire non-redundant unigenes.

2.5. Functional annotation of the assembled unigenes

The unigene sequences were searched using BLASTX against the databases of NR, KEGG, GO, COG, KOG, Pfam and Swiss-Prot to retrieve protein functional annotations based on sequence similarity. High-priority databases (followed by Nr, Swiss-Prot, and KEGG) were selected to determine the direction of the unigene sequences. The best aligning results were used to predict the coding region sequences from unigenes, and the coding sequences (CDs) were translated into amino sequences using the standard codon table. The ESTScan software was used to determine the sequence direction of the unigenes that could not be aligned to any of the above databases.

Gene ontology (GO) terms were assigned to each sequence annotated by BLASTX against the Nr database using the Blast2GO program with an E-value threshold of 1E-5 for further functional categorization. The WEGO software was used to plot the distribution of the GO functional classification of the unigenes. The unigene sequences were also aligned to the COG database to predict and classify possible functions. The unigenes were assigned to KEGG pathway annotations to analyze inner-cell metabolic pathways and the related gene function using BLASTX.

2.6. Analysis of the functional enrichment of DEGs

The FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values were directly used to compare gene expression differences