

## RESEARCH ARTICLE

### Whole Transcriptome Profiling Reveals the ceRNA Network in Chicken T Cells with Stable CD8A Overexpression

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#### ABSTRACT

The CD8 $\alpha$  (CD8A) gene plays an important role in immune function, particularly in T cells. Our previous study demonstrated that CD8A overexpression promotes T-cell activation. Coding and non-coding RNAs are presumed to participate in CD8A-related molecular events in chicken T cells; however, their expression patterns and underlying molecular links remain largely unclear. To address this knowledge gap, we performed whole-transcriptome RNA-seq to analyze mRNA, lncRNA, miRNA and circRNA expression profiles in chicken T cells with CD8A overexpression. The results revealed 1502 significantly differentially expressed (DE) genes, 290 DE-lncRNAs, 23 DE-circRNAs, alongside 27 DE-miRNAs. From our computationally predicted ceRNA interaction dataset, CD8A overexpression correlates with the enrichment of multiple functional pathways, including “positive regulation of cytokine production involved in inflammatory response”, “positive regulation of chemokine-mediated signaling pathway,” and “positive regulation of hormone biosynthetic process”, which may potentially contribute to chicken T-cell activation. Additionally, 7 genes were significantly enriched in the GO terms and KEGG pathways, like HIF1A, KPNA6, AKT3, INPP5K, TASOR, IL20 and AQR. They were immune genes regulated by miR-212-5p, miR-153-5p\_L+1, miR-11987L\_1+1\_1ss8TA, miR-30a-3p, miR-130-3p and PC-3p-865\_3810. The above-mentioned miRNAs showed competitive combinations with additional lncRNAs, such as MSTRG.3834.4, MSTRG.7401.2, MSTRG.2136.8, MSTRG.8713.1, MSTRG.2549.18, MSTRG.7401.2, MSTRG.7401.3 and MSTRG.7882.7 etc., through circRNAs such as cirRNA3035 and cirRNA3926. Consequently, CD8A is associated with T-cell activation and immune responses in chickens, which may be linked to the computationally predicted ceRNA interactions and related pathways.

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#### INTRODUCTION

Poultry is a vibrant and important sector in many countries (Ghaffar, 2025). Precursor T cells develop rapidly in the thymus, gradually differentiating from double-negative CD4-CD8-T cells into double-positive CD4<sup>+</sup>CD8<sup>+</sup>T cells. Through positive and negative selection, an individual positive CD4<sup>+</sup> or CD8<sup>+</sup>T cell bank with complete function and no autoreactivity is

finally formed (Zdrojewicz *et al.*, 2016; Takahama *et al.*, 2017; Ribatti *et al.*, 2019). CD8<sup>+</sup>T cells that migrate to peripheral immune organs target virus-infected cells and induce apoptosis of the target cells by secreting pro-inflammatory cytokines, mediating Fas/FasL interactions, and releasing perforin (Schmidt and Varga, 2018; Wik and Skålhegg, 2022; Mazhar *et al.*, 2025). CD4<sup>+</sup>T cells participate in the clearance of antigens indirectly through modulating other immune cell activity

(Zander *et al.*, 2019). Therefore, T cells are the key cells that exert immune function. Importantly, CD4<sup>+</sup> and CD8<sup>+</sup>T cells more effectively eliminate pathogens only through activation, proliferation, and clonal expansion; otherwise, it may result in T cell exhaustion and later cause a decline in immune function (Wik and Skálhegg, 2022; Yan *et al.*, 2024). Therefore, T cell activation and proliferation can effectively exert the body's immune function.

CD8A, the co-receptor of the T-cell receptor (TCR), can combine with the major histocompatibility complex I (MHC I) molecule in the extracellular region, enhancing the TCR-antigen-MHC triad structural stability, and combine with the tyrosine-activating motif (ITAM) in the intracellular region to regulate T-cell proliferation and activation (Cole and Gao, 2004; Gezer *et al.*, 2024; Dinan *et al.*, 2025). CD8A can bind to MHC class I thymoleukemia antigens (TL) (non-classical) in mice in a TCR-independent manner, thereby generating a new mechanism to regulate T cells via CD8A-MHC Class I (Leishman *et al.*, 2001). Besides, CD8A binds to and interacts with PILR $\alpha$  in mice with CD8A knockout, thus maintaining CD8<sup>+</sup> T cell quiescence (Zheng *et al.*, 2022). The CD8A gene was also identified through transcriptome sequencing of the thymus tissue of immunosuppressed Gushi chickens (Zhai *et al.*, 2020). Studies have shown that the CD8A gene shows high expression within the thymus and T cells in pufferfish (Zhang *et al.*, 2017). In the tumor microenvironment, the CD8A gene also shows an abnormally high expression state in T cells (Lei *et al.*, 2017). According to another study, a haplotype (p.ly111SER) mutation in the CD8A gene showed T cell deficiency in the Roma population of Spain, thereby

affecting the body's immunity (Xu *et al.*, 2014). From the above observations, CD8A has a key effect on modulating immunity and is tightly associated with T cells. In our prior research, T lymphocyte activation was enhanced in CD8A overexpression (Fig. 1A) (Du *et al.*, 2025). Moreover, the CD8A-regulatory gene network remains largely unexplored. Therefore, it is necessary to analyze the CD8A (distributed on the T cell surface) function and its associated regulatory network to better understand chicken immunity.

Many non-coding RNAs-including long non-coding RNAs (lncRNAs), microRNAs (miRNAs), as well as circular RNAs (circRNAs) exert modulatory effects through highly coordinated and precisely orchestrated regulatory networks at the post-transcriptional level (Ma *et al.*, 2018). The whole-transcriptome sequencing analysis is widely employed to elucidate the regulatory mechanisms of molecular networks within chicken (Sohn *et al.*, 2018; Xiao *et al.*, 2022; Li *et al.*, 2024). Moreover, whole-transcriptome sequencing is conducted on T cells, enabling the identification of transcripts of interest (Jivanjee *et al.*, 2022). But research regarding whole-transcriptome sequencing on CD8A gene overexpression within T cells of chicken is lacking.

The present work profiled lncRNA, circRNA, miRNA, and mRNA expression following CD8A overexpression in chicken T cells (Fig. 1B; Fig. 1C). We further predicted potential ceRNA interactions among lncRNAs, mRNAs, circRNAs, and miRNAs. Our results suggest that this molecular network represents a candidate module associated with CD8A expression in chicken T cells. These findings provide new insights into CD8A-related molecular events in chickens.

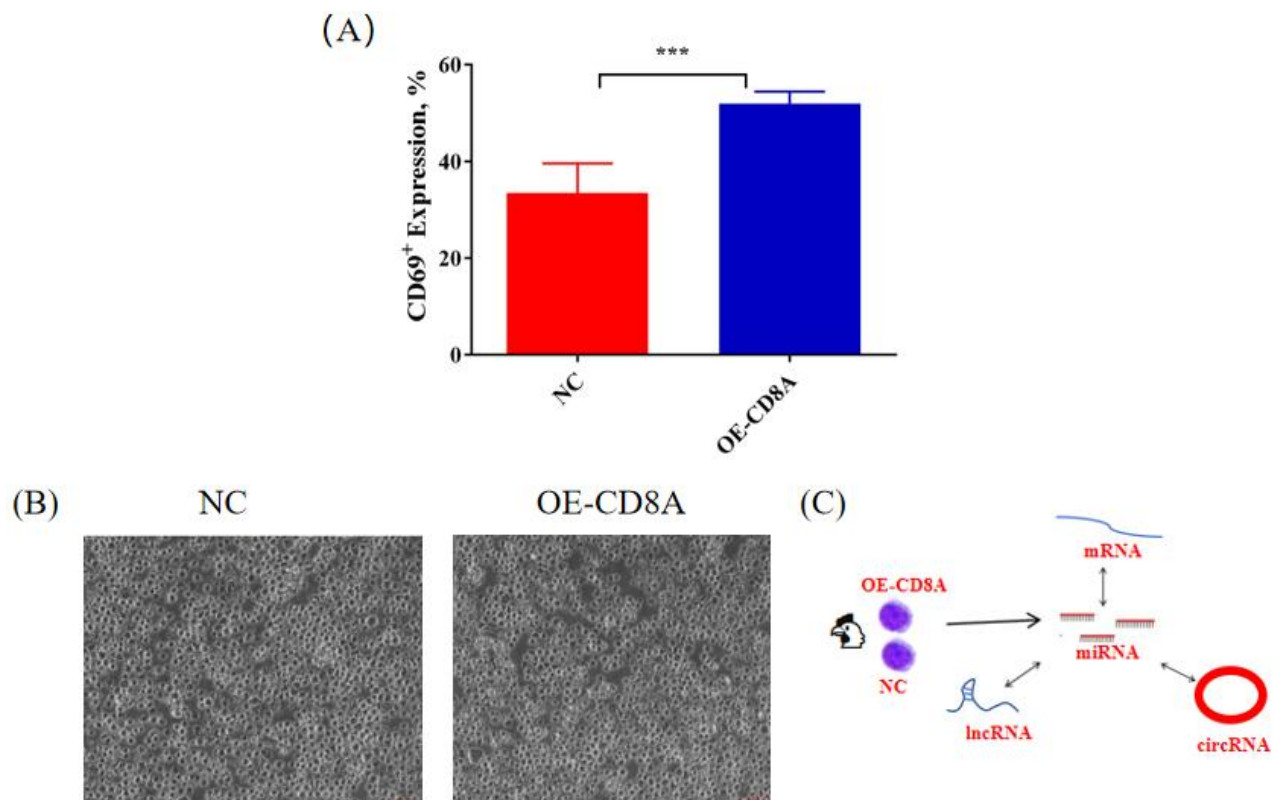


**Fig. 1:** Role of CD8A in T lymphocyte activation. The role of CD8A in (A) T lymphocyte activation was analyzed. (B) Chicken T lymphocyte morphology within the NC and OE-CD8A groups was analyzed. (C) Sketch map showing the experimental flowchart. \*\*\* $P < 0.001$ ; OE-CD8A, T cells with CD8A overexpression; NC, T cells with no-load plasmid.

## MATERIALS AND METHODS

**Ethical statement and experimental animals:** All animal procedures were reviewed and approved by the Animal Care and Use Committee at Kunming University (approval ID: kmu2024042).

**Animal rearing and sampling:** According to our earlier publication (Du *et al.*, 2025), a recombinant expression vector designed to drive high-level CD8A expression was generated and introduced into primary chicken T cells via transfection. These T cells were freshly isolated from thymic tissue of 14 days old six Arbor Acres broiler birds that were provided by Yunnan Agricultural University and cultured. Two experimental groups were established: a negative control group (NC) infected with an empty viral vector, and an overexpression group (OE-CD8A) transduced with the recombinant vector pcADV-EF1-mNeonGreen-CMV-MCS-3xFLAG carrying the full-length CD8A coding sequence. Samples were also harvested from NC and OE-CD8A (three biological replicates per group), immersed in liquid nitrogen at once, and preserved at -80°C before further examination.

**RNA extraction, library construction, and sequencing:** TRIzol reagent (Thermo Fisher) was utilized for extracting total RNA. The total RNA content and quality were later examined with the Bioanalyzer 2100 as well as the RNA 6000 Nano LabChip Kit (Agilent, CA, USA). Ribosomal RNA was removed from the total RNA using the Ribo-Zero Gold ribosomal RNA Removal Kit (Illumina, San Diego, USA). The remaining RNA was cut into short fragments with the NEBNext Magnesium ion RNA Fragmentation Module. cDNA was synthesized from short RNA fragments with SuperScript<sup>TM</sup>II Reverse Transcriptase (Invitrogen, USA). RNase H, *Escherichia coli* DNA polymerase I, and dUTP solution were subsequently utilized to prepare the second cDNA strand. Fragment screening was performed using AMPureXP magnetic beads (300-600 bp). The second DNA strand was treated with thermosensitive UDG enzyme, and then the ligated products were subjected to PCR amplification: 3 min of pre-denaturation at 95°C; 15-s of denaturation at 98°C, 15-s of annealing at 60°C, 30-s of extension at 72°C, for a total of 8 cycles; 5 min of final extension at 72°C. The mean insert fragment length in the cDNA library was 300±50 bp. At last, the Illumina Novaseq<sup>TM</sup>6000 platform was adopted for 2×150 bp paired-end sequencing.

For circRNA, the steps of total RNA isolation, content and quality detection and ribosomal RNA removal were the same as described above. After removing ribosomal RNA, the remaining RNA was subjected to 30 min of RNase R (Epicentre, USA) treatment under 37°C for degrading linear RNA while enriching circular RNA. After enrichment, circular RNA was fragmented, reverse transcribed, second-strand synthesized, A-tailed, ligated with adapters, and subjected to fragment screening, UDG enzyme treatment and PCR amplification as described above. The mean insert fragment length was 300±50 bp in the eventual cDNA library, which then underwent sequencing using 2×150bp paired-end reads with the Illumina Novaseq<sup>TM</sup> 6000 platform.

For miRNA, a miRNA library was established with TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, USA) in line with specific protocols. Briefly, total RNA (5µg) was introduced into 3' and 5' adapter-ligated RNA of the miRNA library. Subsequently, reverse transcription was performed to generate cDNA, which was then amplified prior to sequencing with the Illumina HiSeq 2000/2500 platform (LC-Bio, Hangzhou, China), yielding 50-bp single-end reads.

**Quality control:** Cutadapt (v 1.9) was adopted for filtering raw reads, yielding high-quality clean reads, mainly by removing reads that contained adapters, polyA/polyG, low-quality bases, and over 5% of unknown nucleotides (N). For miRNA, the ACGT101-miR (v 4.2) analysis platform was used to conduct a comprehensive bioinformatics analysis on miRNA sequencing results. After removing low-quality sequences and 3' adapter sequences from raw reads, sequences with an excessively high proportion of N, an excessively low average quality value, and those that were too short after trimming were eliminated to obtain high-quality Clean Data. We selected sequences containing 18-26 nucleotides for subsequent analysis. To eliminate interference from non-target small RNAs, the sequences were aligned with the mRNA, RFam, and Rfam databases for filtering and the remaining sequences were used as candidate miRNA datasets.

**Alignment with reference genome:** Clean reads obtained were aligned against the chicken reference genome ([ftp://ftp.ensembl.org/pub/release-107/fasta/gallus\\_gallus/dna/](ftp://ftp.ensembl.org/pub/release-107/fasta/gallus_gallus/dna/)) with HISAT2 (v 2.2.1). UMI-tools software was used to remove sequence preferences and amplification noise. For circRNAs, clean reads obtained were aligned to the chicken reference genome with TopHat2 software (v 2.0.4). The reads that were not mapped were re-mapped using TopHat-Fusion software (v 2.1.0) for subsequent identifying circRNA. For miRNA, the candidate miRNA sequences were mapped to miRBase 22.1 and the chicken genome.

**Transcript reconstruction, novel transcript identification, and annotation:** Transcript assembly using StringTie (v 2.1.6), combined with HISAT2, can detect novel genes or novel splicing isomers of known genes. All assembled transcripts were compared with the chicken reference genome using gffcompare (v 0.9.8) and classified into 12 categories. Transcripts of type codes i, j, o, u and x are considered new transcripts for subsequent lncRNA prediction.

**lncRNA, circRNA and miRNA identification:** To identify lncRNAs, transcripts overlapping with known mRNA/lncRNA and those < 200bp long were removed. Then, using CPC0.9-r2 and CNCI2.0 under default parameters, the new transcripts showing coding potential were predicted. The transcripts satisfying CPC score < 0.5 and CNCI score < 0 were reserved as the new lncRNAs. Meanwhile, the rest transcripts related to class codes i, j, o, u, or x were considered novel lncRNAs.

To identify circRNA, Candidate circRNAs were identified using the CIRCexplorer2 pipeline (v 2.2.6). Back-spliced junction (BSJ) signals were extracted from

TopHat-Fusion chimeric alignment outputs. To reduce false-positive predictions, we applied the following filtering criteria: (1) circRNAs supported by at least two unique BSJ-spanning reads; (2) removal of multi-mapping ambiguous reads; (3) reasonable genomic span limits for circRNA candidates. CircRNA expression levels were calculated based on the number of BSJ-supporting reads. Circular RNA candidates identified across all samples were integrated to generate a non-redundant, sample-unified circular RNA catalog.

To identify miRNAs: ACGT101-miR (v 4.2) was employed for removing repeat sequences, adapter dimers, low-complexity, junk and overlapping RNA families (tRNA, rRNA, snRNA, snoRNA). Subsequently, miRBase 22.1 was utilized for locating known and novel miRNA sequences (18-26 bp long) within precursors. The terms prefixed with “miRs” stand for mature miRNAs, and those prefixed with “PC” stand for novel miRNAs.

**Gene expression quantification:** StringTie was employed for assembling reads in every sample after alignment under default parameters. Later, the transcriptomes were combined with gffcompare, yielding a complete transcriptome. Transcript levels were calculated with StringTie and ballgown, with FPKM (fragments per kilobase of exon per million mapped reads) as a quantification index.

For CircRNA, the number of trans-splicing reads was standardized by SRPBM (the number of trans-splicing reads per billion mapped reads divided by read length) to quantitatively compare trans-splicing events among different RNA sequencing datasets. The overall read number aligned to the reference genome for each sample was counted, and the number of reads across trans-splicing sites was standardized by the overall aligned read number (in billions) and read length. The SRPBM value for each circular RNA was calculated through a Perl script as expression abundance. The formula for SRPBM calculation is the circular read quantity / the overall aligned read number (in billions) / read length.

For miRNA, ACGT101-miR was adopted for identifying miRNAs and calculating miRNA levels. Through statistical analysis of expression data, the associations between miRNA expression features within and between groups, as well as differentially expressed miRNAs, were analyzed. The norm value (normalized from the raw read count of miRNA) was used as the measurement index of miRNA levels, while miRNA levels within diverse samples were statistically analyzed. Simply put, the norm value can be understood as the expression level of miRNA.

**Differential expression analysis:** For mRNA, lncRNA, and miRNA, we conducted differential analysis with DESeq2 software using the  $P\text{-value} < 0.05$  and  $|\log_2\text{fold change}| \geq 1$  criteria. For CircRNA, differential analysis of circRNAs was conducted with edgeR software upon  $P\text{-value} < 0.05$  and  $|\log_2\text{fold change}| \geq 1$  criteria.

Screening potential target genes of DE-lncRNAs/DE-circRNAs/DE-miRNAs and functional annotations.

Using the Python script, coding genes 100kb up- and downstream were chosen to be cis-target genes of

lncRNAs. For DE-lncRNAs, their target genes were conducted GO and KEGG analyses.  $P < 0.05$  stood for statistical significance.

miRanda (version 3.3a) and TargetScan (version 5.0) were utilized to predict DE-circRNAs as miRNA targets (miRBase, <http://www.mirbase.org>) in accordance with the TargetScan\_score  $\geq 50$  and miRanda\_Energy  $< -10$  thresholds. Host coding genes of DE-circRNAs conducted GO and KEGG enrichment analyses, with  $P < 0.05$  indicating statistical significance.

Similarly, miRanda (version 3.3a) and TargetScan (version 5.0) were employed to predict target genes of DE-miRNAs. Target genes showing context score percentiles  $< 50$  were eliminated in TargetScan, while those showing maximum free energy (Max Energy)  $> -10$  were eliminated in miRanda. Thereafter, for DE-miRNAs, their target genes were performed GO and KEGG analyses, with  $P < 0.05$  suggesting statistical significance.

**ceRNA Regulatory network prediction:** To explore the associations of DE-lncRNAs, DE-circRNAs, DE-miRNAs and DE-genes, a circRNA/lncRNA-miRNA-mRNA regulatory network was established based on the ceRNA hypothesis. Using TargetScan and miRanda, miRNA-mRNA, miRNA-lncRNA, and miRNA-circRNA pairs were calculated. Finally, the circRNA/lncRNA-miRNA-mRNA network was visualized with Cytoscape (v 3.9.1).

## RESULTS

**Quality control and mapping statistics of RNA-seq data:** Six cDNA libraries corresponding to the NC and OE-CD8A groups were generated in this study. Each sample yielded no fewer than one hundred fifteen million raw sequencing reads. Following adapter trimming and filtering of low-quality reads, a minimum of one hundred three million clean reads were retained per sample. Across all libraries, the average Q20 and Q30 values of clean reads reached 99.82 % and 97.32 %, respectively, with an average GC content of 47.6 %. All six libraries were mapped against the chicken reference genome, resulting in mapping rates ranging from 86.07 % to 91.28 % (Table 1, Du *et al.*, 2025).

**DE-genes expression:** To investigate the RNA expression profiles associated with T cell activation under CD8A overexpression, we conducted whole-transcriptome RNA-seq on T cells derived from CD8A-overexpressing (OE-CD8A) as well as NC experimental groups. As revealed by principal component analysis (PCA), mRNA expression patterns were clearly separated between the OE-CD8A and NC groups within chicken T cells (Fig. 2A). All six samples had consistently high sequencing quality (Fig. 2D). There were altogether 1502 DE-genes detected within T cells between the OE-CD8A and NC, which included 777 with upregulation whereas 725 with downregulation (Fig. 2C). FPKM-based hierarchical clustering revealed clear separation between sample groups, whereas samples within the same group exhibited tight clustering (Fig. 2B). GO alongside KEGG analysis was carried out for elucidating biological functions of the DE-genes. We subsequently conducted GO enrichment on these 1502 DE genes and 165 GO

terms were enriched, where about 110 terms were enriched into biological process (BP) related to “defense response”, “humoral immune response,” and “CD8-positive, alpha-beta T cell differentiation involved in immune response,” etc. Moreover, 20 cellular component (CC) terms were closely associated with “secretory dimeric IgA immunoglobulin complex”, “dimeric IgA immunoglobulin complex,” and “secretory IgA

immunoglobulin complex,” etc. Further, 35 molecular function (MF) terms were associated with “IgA binding”, “interleukin-8 receptor binding,” and “creatine kinase activity etc. (Fig. 2E). The 465 DE-genes were subjected to KEGG analysis, with 5 pathways being significantly enriched ( $P < 0.05$ ), which were related to “Toll-like receptor signaling pathway”, “Cellular senescence,” and “Glutathione metabolism” (Fig. 2F).

**Table I:** Quality control and mapping statistics of RNA-seq data

| Sample    | Raw Data    |          | Clean Data  |          | Q20%  | Q30%  | GC%   | Total_map (%) |
|-----------|-------------|----------|-------------|----------|-------|-------|-------|---------------|
|           | Reads       | Base (G) | Reads       | Base (G) |       |       |       |               |
| NC_1      | 121,225,140 | 18.18    | 109,672,232 | 16.45    | 99.83 | 97.41 | 47    | 91.23%        |
| NC_2      | 120,848,194 | 18.13    | 107,018,196 | 16.05    | 99.82 | 97.35 | 47    | 90.91%        |
| NC_3      | 115,348,956 | 17.30    | 103,645,522 | 15.55    | 99.80 | 97.31 | 48    | 86.07%        |
| OE-CD8A_1 | 121,449,454 | 18.22    | 108,226,660 | 16.23    | 99.81 | 97.20 | 48    | 90.19%        |
| OE-CD8A_2 | 119,529,366 | 17.93    | 105,944,852 | 15.89    | 99.81 | 97.32 | 48    | 91.01%        |
| OE-CD8A_3 | 118,702,502 | 17.81    | 106,337,262 | 15.95    | 99.84 | 97.30 | 47.50 | 91.28%        |

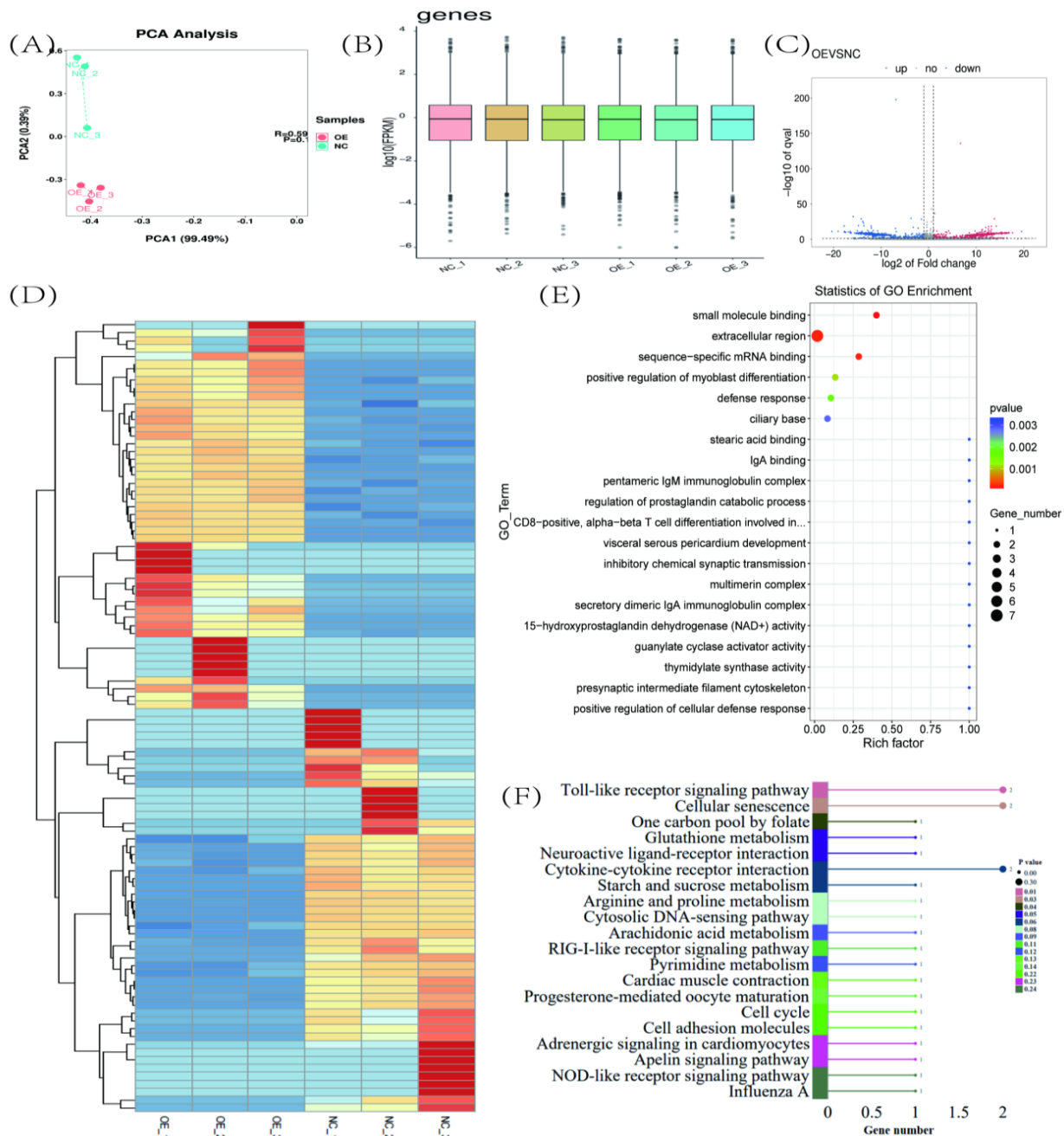


**Fig. 2:** Differential expression profiling following CD8A overexpression within chicken T cells. (A) PCA plot showing mRNA levels within six samples. (B) Violin plot depicting the distribution of expression levels for all detected genes. (C) Volcano plot and (D) hierarchical heatmap of DE-genes between the OE-CD8A and NC groups. Orange and blue denote up- and downregulation separately. (E) GO and (F) KEGG pathway analyses of DE-genes.

**DE-lncRNA expression:** We analyzed the mRNA and lncRNA lengths. Our results showed that mRNAs and lncRNAs > 1000 bp long constituted 99.23 and 77.87% separately (Fig. 3A). Their open reading frame (ORF) length distribution was examined. As discovered, 87.4% of lncRNAs had ORFs that were 0-150 amino acids long (Fig. 3B), while 67.2% of mRNAs had ORFs that were 100-600 amino acids long (Fig. 3C). mRNA levels and transcript count both exceeded those of lncRNAs (Fig. 3D). There were altogether 290 DE-lncRNAs detected within T cells of OE-CD8A and NC, which included 166 with upregulation whereas 124 with downregulation (Fig. 3E). The FPKM-based hierarchical clustering revealed clear separation between sample groups, whereas samples within the same group exhibited tight clustering (Fig. 3F). Further,

GO annotation was carried out on genes regulated by DE-lncRNAs, with 56 GO terms enriched, where about 43 terms were associated with BPs primarily related to the “positive regulation of B cell activation”, “hepatic immune response” and “positive regulation of innate immune response” etc. In the CC, 4 terms were mostly related to “interleukin-6 receptor complex”, “sperm connecting piece” and “eukaryotic translation initiation factor 2 complex” etc. In the MF category, 9 significant terms were enriched, primarily associated with “thyroid hormone binding,” “retinal dehydrogenase activity,” and “chemokine receptor activity” (Fig. 3G). KEGG analysis was carried out, with 6 closely related pathways identified, primarily associated with “Linoleic acid metabolism”, “Selenocompound metabolism” and “Hedgehog signaling pathway” (Fig. 3F).

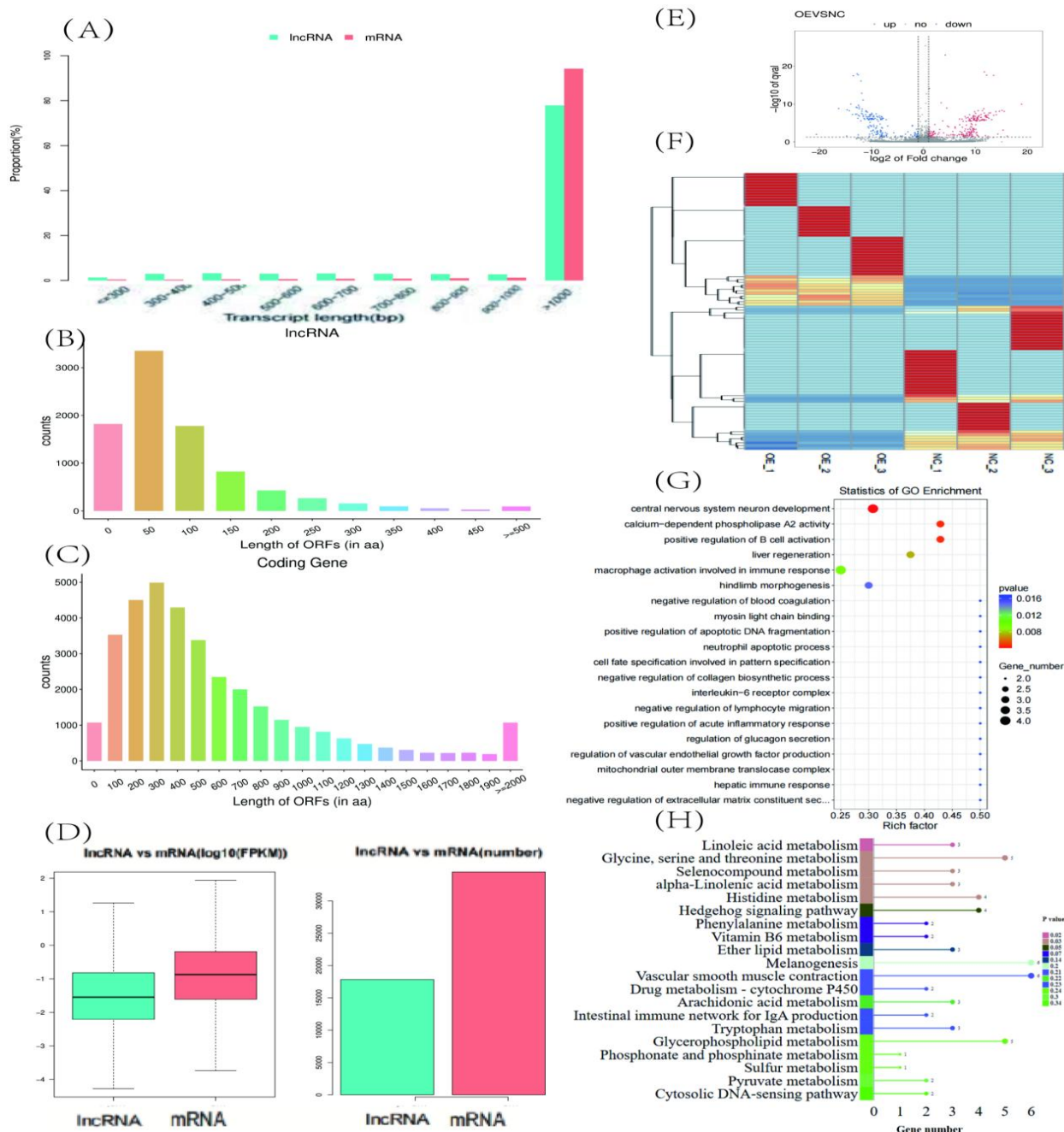


**Fig. 3:** Differential expression analysis of lncRNAs following CD8A overexpression in chicken T cells. (A) lncRNA and mRNA lengths. (B) ORF length distribution in lncRNAs and (C) mRNAs. (D) Comparative summary of expression levels and transcript counts for lncRNAs and mRNAs. (E) Volcano plot and (F) hierarchical clustering heatmap illustrating DE-lncRNAs in OE-CD8A vs. NC; orange and blue stand for up- and downregulated DE-lncRNAs separately. (G) GO and (H) KEGG analyses of DE-lncRNAs.

**DE-circRNAs expression:** There were altogether 23 DE-circRNAs detected within T cells between OE-CD8A and NC, which included 16 showing upregulation whereas 7 exhibiting downregulation (Fig. 4A). Fig. 4B displays FPKM cluster analysis results. Samples between groups were separate, while samples within groups were clustered together. As observed from the scatter plot, the mean DE-circRNA levels were greatly different between groups (Fig. 4C). Next, host genes of DE-circRNAs were subjected to GO annotation and 129 GO terms were enriched, where about 92 closely related BP terms were associated with

“negative regulation of interleukin-12 production”, “chronic inflammatory response” and “interleukin-1-mediated signaling pathway” etc. In the CC, 18 closely related terms were identified, mainly associated with “secretory granule”, “extracellular matrix” and “mitochondrial transcription factor activity” etc. There were 19 terms significantly related to MFs, mainly involving “interleukin-1 receptor activity”, “interleukin-1, type I, activating receptor activity” and “interleukin-1 receptor activity” etc. (Fig. 4D). According to KEGG analysis, 1 pathway, namely, “ECM-receptor interaction” was co-enriched ( $P < 0.05$ ) (Fig. 4E).

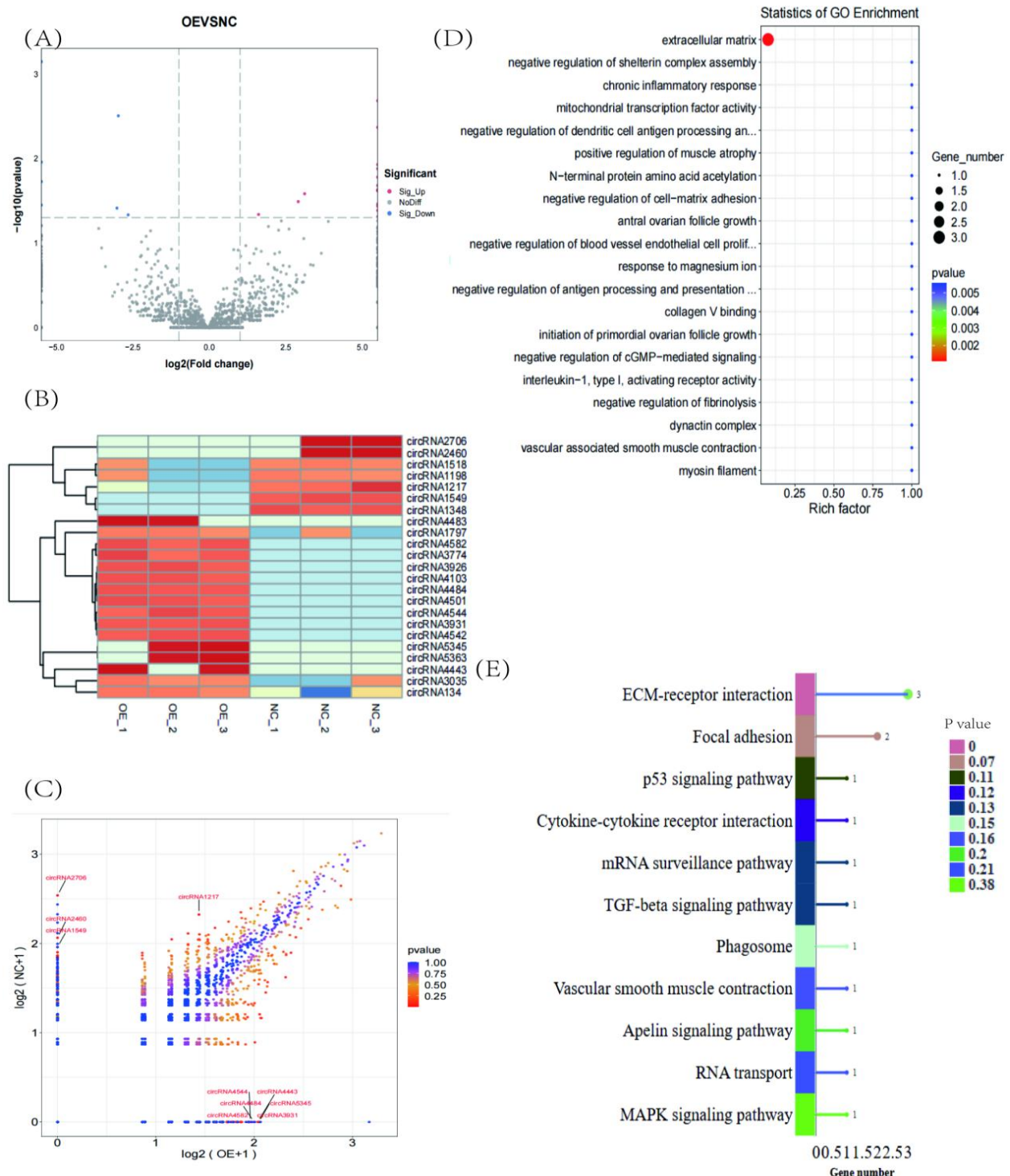


**Fig. 4:** Differential analysis of circRNAs after CD8A overexpression within chicken T cells. (A)Volcano plot and (B) hierarchical clustering heatmap showing DE-circRNAs for OE-CD8A vs. NC. Orange and blue stand for up- and downregulation separately. (C) Scatter plot showing DE-circRNAs. (D) GO and (E) KEGG analysis of DE-circRNAs.

**Expression of DE-miRNAs:** There were altogether 27 DE-miRNAs detected within T cells between OE-CD8A and NC, which included 11 with upregulation and 16 with downregulation (Fig. 5A). Fig. 5B displays the FPKM cluster analysis results of DE-miRNAs; the FPKM-based hierarchical clustering revealed clear separation between sample groups, whereas samples within the same group exhibited tight clustering. To explore their possible functions, target genes for those identified miRNAs were predicted. Next, GO annotation was carried out on target genes of DE-miRNAs, with 293 GO terms enriched, including 172 closely related to

BPs such as “activation of innate immune response”, “T cell activation,” and “immune response,” etc. In the CC, 73 closely related terms were identified to be associated with “membrane”, “plasma membrane,” and “Golgi apparatus” etc. Meanwhile, 48 significantly enriched MF terms were identified to be associated with “GTPase activity”, “GTP binding,” and “transferase activity,” etc. (Fig. 5C). Target genes of DE-miRNAs were subjected to KEGG analysis, which revealed co-enrichment of 22 pathways ( $P < 0.05$ ), such as “Wnt signaling pathway”, “Metabolic pathways,” and “Autophagy - animal,” etc. (Fig. 5D).

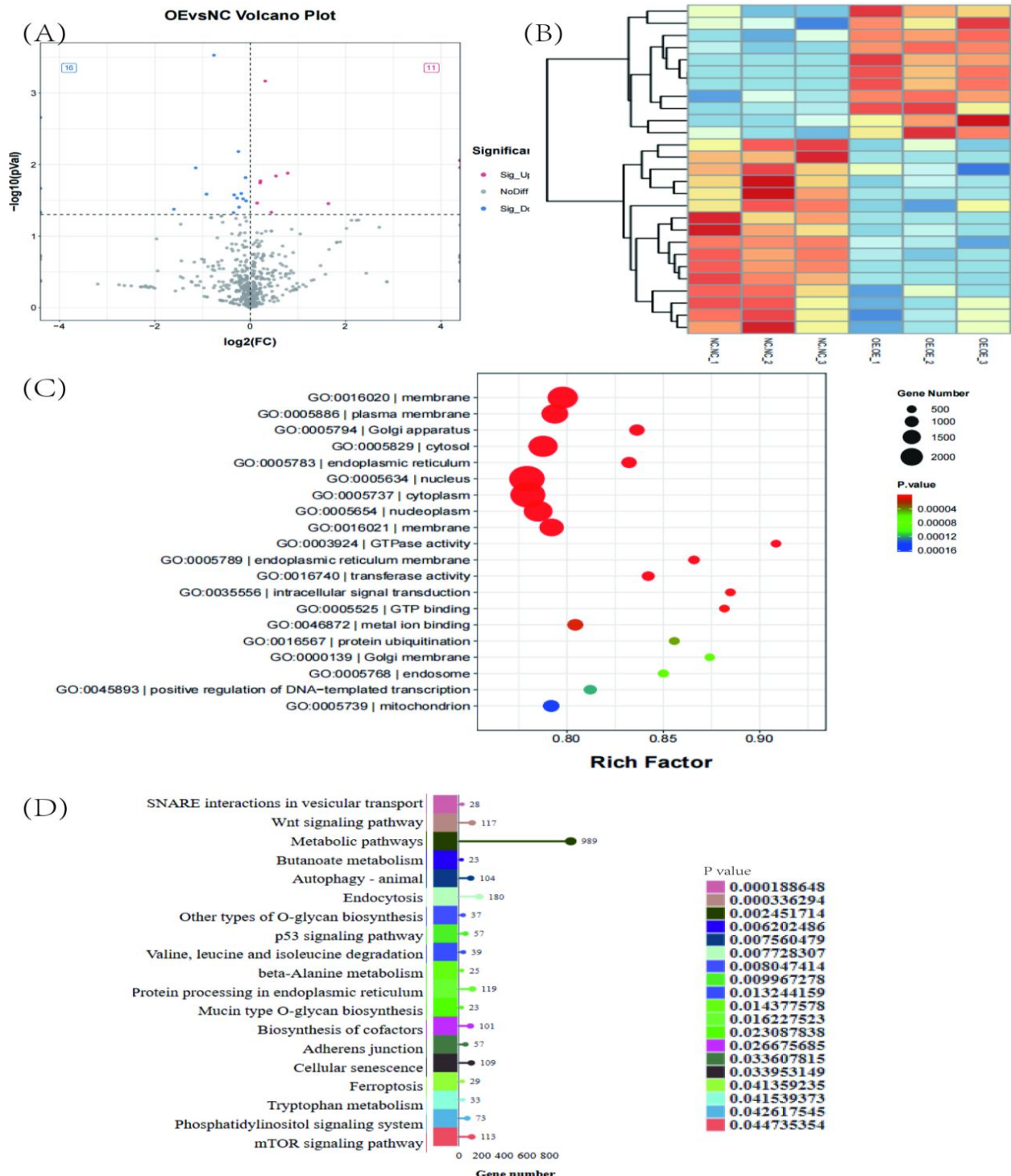


**Fig. 5:** Differential miRNA analysis following CD8A overexpression in chicken T cells. (A) Volcano plot and (B) hierarchical clustering heatmap showing DE-miRNAs for OE-CD8A vs. NC. Orange and blue denote up- and downregulation separately. (C) GO and (D) KEGG analyses of DE-miRNAs.

**ceRNA Network establishment:** miRNA-mRNA, circRNA-miRNA and lncRNA-miRNA interactions were predicted using TargetScan and miRanda, identifying 788, 20 and 1,310 interaction pairs, respectively. Regarding the lncRNA/circRNA-miRNA-mRNA interaction networks, 270 circRNA-miRNA-mRNA interactions were detected, involving 7 circRNAs, 18 miRNAs and 55 mRNAs (Fig. 6A). In total, there were 14,870 lncRNA-miRNA-mRNA interactions detected, involving 287 lncRNAs, 437 mRNAs, alongside 108 miRNAs (Fig. 6B). According to mRNAs related to the lncRNA/circRNA-miRNA-mRNA network, we carried out GO as well as KEGG analysis. Targeted mRNAs among the lncRNA/circRNA-miRNA-mRNAs were conducted GO analysis, revealing “positive regulation of chemokine-mediated signaling pathway”, “positive regulation of cytokine production involved in inflammatory response”, “positive regulation of hormone

biosynthetic process,” etc. (Fig. 6C). No pathway was enriched based on KEGG analysis. To detect the potential ceRNA transcript-interaction associations, 7 genes that were significantly enriched in the GO and KEGG enrichment analyses were selected and identified as immunity candidate genes, including hypoxia inducible factor 1 subunit alpha (HIF1A), karyopherin subunit alpha 6 (KPNA6), AKT serine/threonine kinase 3 (AKT3), inositol polyphosphate-5-phosphatase K (INPP5K), transcription activation suppressor (TASOR), interleukin 20 (IL20) and aquarius intron-binding spliceosomal factor (AQR). Consequently, ceRNA networks were established for the 7 genes separately, which involved circRNA-miRNA-mRNA and lncRNA-miRNA-mRNA interactions, including 6 miRNAs, 2 circRNAs, 73 lncRNAs and 113 interaction associations (Fig. 7).

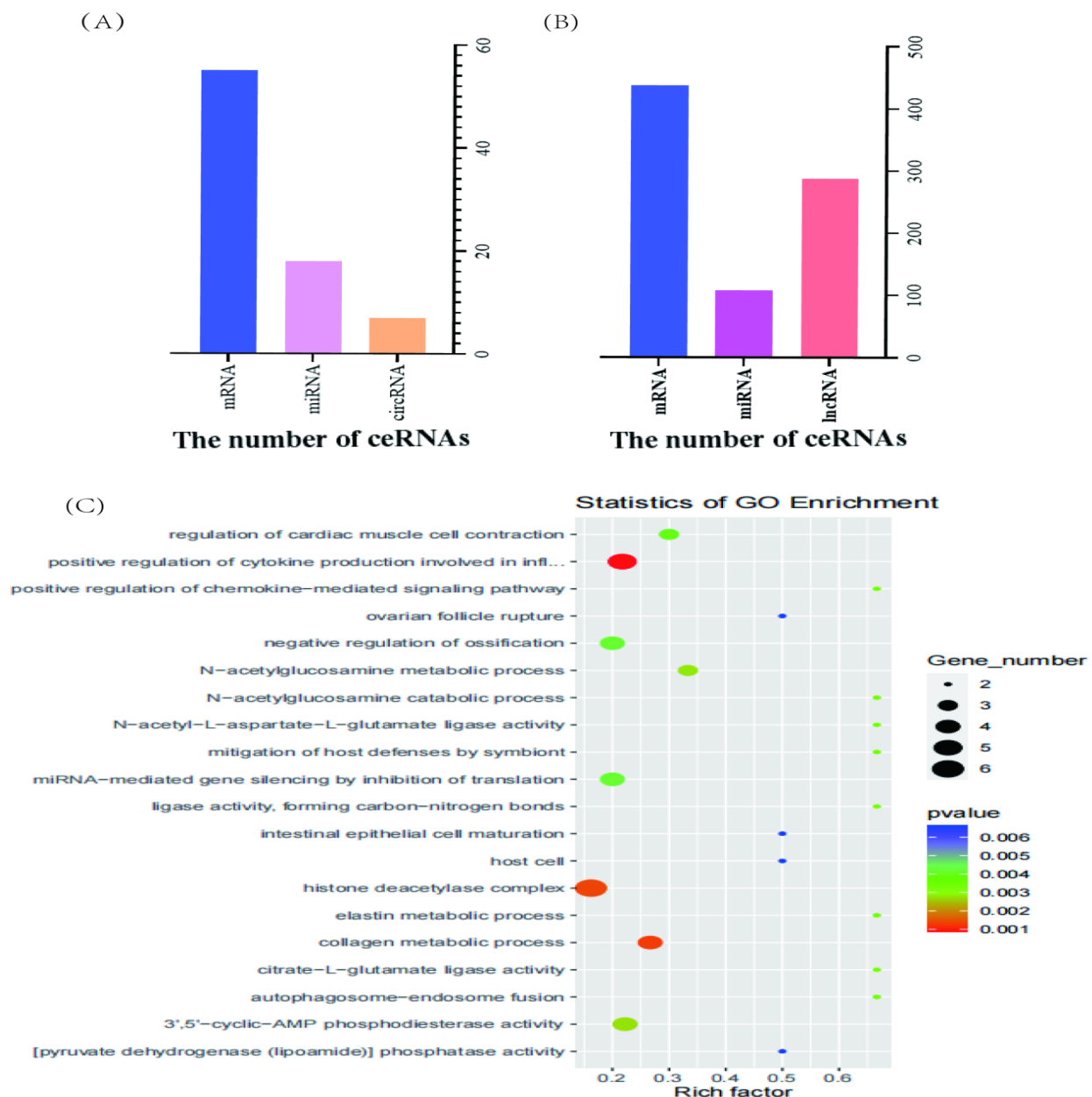


Fig. 6: CeRNA network predictions derived from DE-RNAs. (A) The mRNA, miRNA, and lncRNA numbers related to lncRNA-miRNA-mRNA interactions. (B) The mRNA, miRNA, and circRNA numbers related to circRNA-miRNA-mRNA interactions. (C) GO annotation of mRNAs within the ceRNA regulatory network.

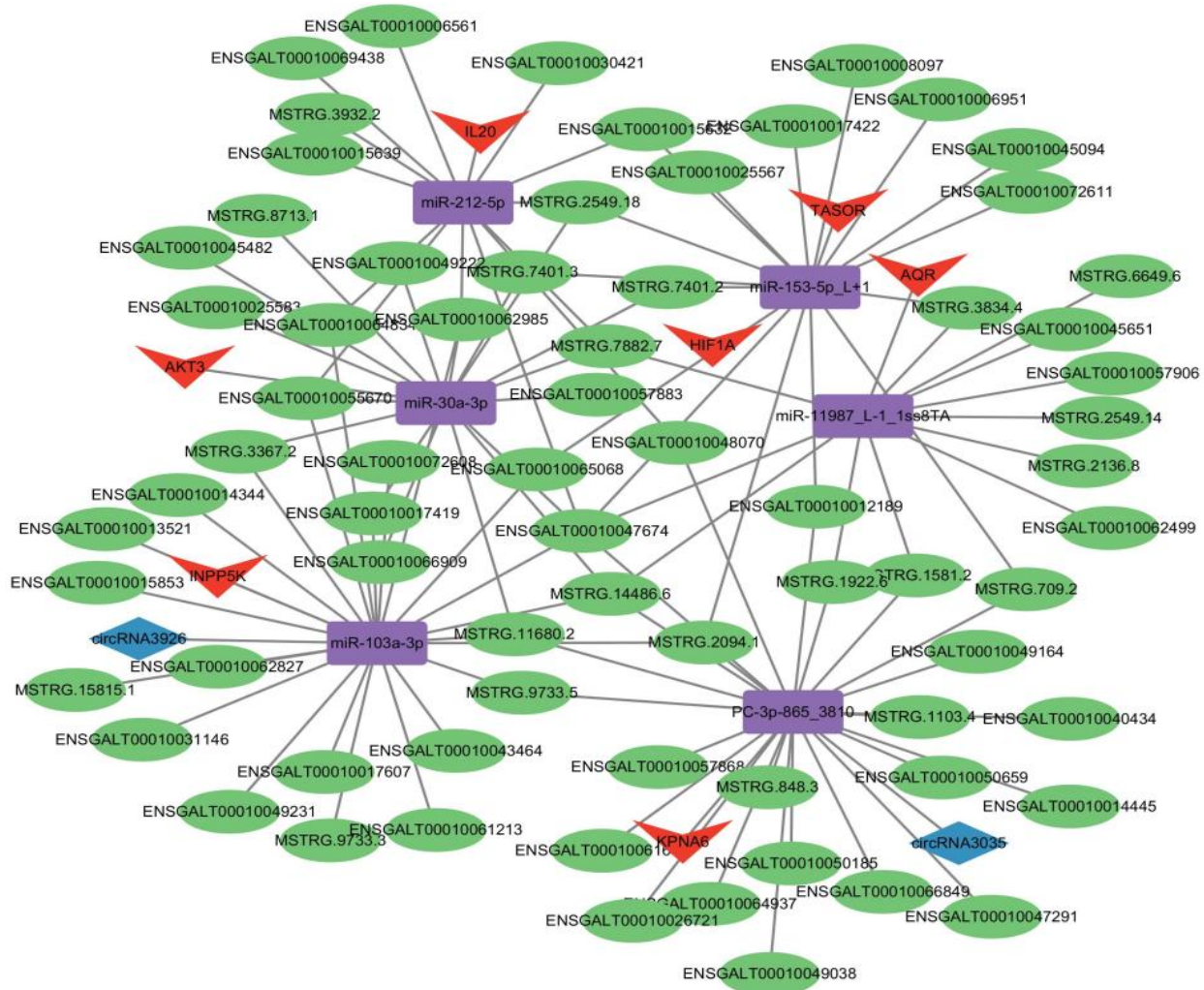


**Fig. 7:** The ceRNA regulatory network of circRNA/lncRNA-miRNA-mRNA interactions closely related to candidate genes within chicken T cells. Green, purple, blue, and red stand for lncRNAs, miRNAs, circRNAs, and mRNAs separately.

## DISCUSSION

CD8A has an important effect on cell-mediated immunity and T-cell growth (Suetake *et al.*, 2007). In our prior work, CD8A overexpression significantly promoted activation of T cells in chickens (Du *et al.*, 2025). Consequently, CD8A regulates immune function and T cells in chickens. In chickens, T cells are a key component of cellular immunity and are regulated at multiple levels by genes encoding proteins, ncRNAs, and transcription factors. So far, the spatiotemporal expression profile of CD8A interference and its functional modulation via lncRNAs, miRNAs and circRNAs has been partially characterized during chicken T cell development (Du *et al.*, 2026). In contrast, the consequences of CD8A overexpression on this process, particularly the associated post-transcriptional regulatory networks governed by these non-coding RNAs, remain entirely unexplored. The present work performed integrated whole-transcriptome RNA-seq for comprehensively profile dynamic changes in mRNA, lncRNA, circRNA and miRNA expression following stable CD8A overexpression in primary chicken T cells. In addition, we systematically reconstructed co-expression and interaction networks between non-coding RNAs (ncRNAs) and mRNAs to identify candidate regulatory axes governing CD8A-dependent T-cell development.

In this study, we first conducted whole-transcriptome RNA-seq of T cells in the OE-CD8A and NC groups, identifying 1502 DE-genes, 290 DE-lncRNAs, 23 DE-circRNAs, alongside 27 DE-miRNAs. GO as well as KEGG analysis of DE-genes revealed the significant enrichment of these genes in pathways including “CD8-positive, alpha-beta T cell differentiation involved in immune response”, “Glutathione metabolism”, “Toll-like receptor signaling pathway,” etc. The “CD8-positive, alpha-beta T cell differentiation involved in immune response” plays a crucial role in immune function. Studies have pointed out that interference with CD8A expression suppresses T cell growth and promotes their apoptosis (Du *et al.*, 2024). CD8A exerts an essential effect on cell-mediated immunity and T-cell development (Suetake *et al.*, 2007). DE-gene, such as Eomesodermin (EOMES) was significantly enriched in “CD8-positive, alpha-beta T cells differentiation involved in immune response”. EOMES regulates T cells development and influences T cell-mediated intestinal inflammatory responses (Xie *et al.*, 2026). EOMES has a key effect on the growth and maturation of natural killer (NK) cells, and on proper T cell differentiation (Pearce *et al.*, 2003; Cruz-Guilloty *et al.*, 2009). EOMES is also critical for T cell homeostasis and survival (Banerjee *et al.*, 2010). The “Toll-like receptor signaling pathway” can directly modulate T cell

proliferation, activation, differentiation and effector function across diverse physiological contexts (Duan *et al.*, 2022). DE-gene, such as IL8L2 and CCL4 were significantly enriched in the “Toll-like receptor signaling pathway”. Previous studies have shown that IL8L2 was significantly overexpressed in spleen tissue from immunosuppressed chicks through transcriptome analysis (Guo *et al.*, 2020). CCL4 is widely reported to initiate T cell migration, activation and differentiation (Sallusto *et al.*, 1999; Tang and Cyster, 1999). “Glutathione metabolism” is tightly associated with T cell proliferation and activation (Suthanthiran *et al.*, 1990; Yan *et al.*, 2010). DE-genes like ENSGALG00010008076 in “Glutathione metabolism” were involved in these pathways. However, there are currently no reports related to the ENSGALG00010008076 gene, and further investigation is still needed. Consequently, such pathways probably influence chicken immunity. Therefore, these significantly enriched signaling pathways and genes influence T cell growth and chicken immunity. From the above findings, CD8A modulates these pathways to influence T cell function in chickens.

miRNA-lncRNA-mRNA and miRNA-circRNA-mRNA networks were constructed for T cells using DE-lncRNAs, DE-genes, DE-circRNAs, and DE-miRNAs. To explore the possible biological functions involved in ceRNA networks, the DE-mRNAs embedded within these networks were subjected to functional enrichment. From GO analysis of candidate genes related to lncRNA/circRNA-miRNA-mRNAs, “positive regulation of cytokine production involved in inflammatory response”, “positive regulation of hormone biosynthetic process”, “positive regulation of chemokine-mediated signaling pathway,” etc., were identified. To examine the possible ceRNA transcript-interaction associations, 7 genes closely related to GO and KEGG pathways were selected and identified as immunity candidate genes, including HIF1A, KPNA6, AKT3, INPP5K, TASOR, IL20, and AQR. HIF1A contributes to the inflammatory response by regulating immune cell activity, especially macrophage and T cell activity; besides, a HIF1A inhibitor mitigated infiltration of inflammatory cells in the experimental autoimmune myocarditis (EAM) model, which probably becomes a candidate therapeutic target (Hua *et al.*, 2020). KPNA6 was closely related to influenza A virus infection and can exert antiviral immune effects by inhibiting viral replication (Huang *et al.*, 2024). AKT3 significantly activates macrophages (Vergadi *et al.*, 2017). Moreover, MiR-150 regulates T cell activity through AKT3 (Sang *et al.*, 2016). INPP5K can regulate T-cell infiltration through DNA methylation and has become an important prognostic marker and target for gynecological cancers (Shang *et al.*, 2021). TASOR can be antagonized by the Vpx protein in human immunodeficiency virus type 2 (HIV-2) or by the Vpr protein delivered by lentivirus (Vauthier *et al.*, 2022) and thereby may be related to the host’s innate immunity to defend against the virus. IL-20 is closely related to immunomodulation and inflammatory reactions (Conti *et al.*, 2003). AQR mice are tightly associated with T cell development (Vermeer *et al.*, 1988). Therefore, these genes have a key effect on immunity and T-cell functions. Consequently, CD8A correlates with these immune

candidate genes, which may be involved in modulating chicken T-cell activity and immunity. At last, those 7 significant DE-genes were selected for constructing the ceRNA network model involving 6 miRNAs, 2 circRNAs, 73 lncRNAs, alongside 113 interaction associations. miR-212-5p, miR-153-5p\_L+1, miR-11987L\_1+1\_1ss8TA, miR-30a-3p, and miR-130-3p, etc. were detected in our established ceRNA network, with some affecting immune function. As reported in some research, miR-212-5p has a key effect on the immune asthma microenvironment (Jia *et al.*, 2024). Other studies have found that miR-30a-3p is correlated with immunity (Manai *et al.*, 2023). The miR-130-3p targets EPH receptor B4 (EPHB4) to enhance MTX-mediated hepatocellular carcinoma cell immune killing (Shao *et al.*, 2021). Moreover, the function of miR-130-3p was inhibited by the RNA circRNF217, thereby enhancing the antibacterial effect and innate immunity of bony fish (Zheng *et al.*, 2022). To date, no studies have reported on miR-153-5p\_L+1 and miR-11987L\_1+1\_1ss8TA, nor on their potential roles in immune regulation and T cell biology. To date, there are no published reports regarding miR-153-5p\_L+1 and miR-11987L\_1+1\_1ss8TA, and their roles in immune regulation and T-cell biology remain uncharacterized. These microRNAs are therefore candidate miRNAs associated with CD8A-related T-cell processes; future functional validation is needed to assess any possible influence on T-cell activity and immunity.

However, the ceRNA interactions reported in this article are based on the bioinformatics prediction results obtained from whole transcriptome sequencing. Further experiments, such as dual luciferase reporter assays for verifying miRNA target binding, are needed to be conducted in the future to further elucidate the actual regulatory mechanism underlying chicken T cell immunity.

**Conclusions:** Collectively, HIF1A, KPNA6, AKT3, INPP5K, TASOR, IL20 and AQR represent key immune-related genes that are associated with CD8A and may be linked to chicken T-cell functions. They are modulated by miR-212-5p, miR-153-5p\_L+1, miR-30a-3p, miR-130-3p, and PC-3p-865\_3810, etc. Such miRNAs show competitive binding to additional lncRNAs, like MSTRG.3834.4, MSTRG.7401.2, MSTRG.2136.8, MSTRG.8713.1, MSTRG.2549.18, MSTRG.7882.7, etc., and to circRNAs, including circRNA3035 and circRNA3926. This sheds novel light on the mechanism of CD8A in affecting chicken T-cell growth and immunity.

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**Availability of Data and Materials:** All data supporting our findings are included in the manuscript. The RNA sequencing data for this study can be found in the NCBI Sequence Read Archive (SRA) under Bioproject: PRJNA1378469 and PRJNA1177976.

**Author contributions:** YD, WH, CG, DF, XZ Conception and design of study; JR, YD Analysis and/or interpretation of data; YD, FSA, KW drafting the manuscript; YD, MSI, JR Critical review/revision; All authors have read and agreed to the published version of the manuscript.

## REFERENCES

- Banerjee A, Gordon SM, Intlekofer AM, et al., 2010. Cutting edge: The transcription factor eomesodermin enables CD8<sup>+</sup> T cells to compete for the memory cell niche. *Journal of Immunology* 185:4988-4992.
- Cole DK and Gao GF, 2004. CD8: adhesion molecule, co-receptor and immuno-modulator. *Cellular and Molecular Immunology* 1:81-88.
- Conti P, Kempuraj D, Frydas S, et al., 2003. IL-10 subfamily members: IL-19, IL-20, IL-22, IL-24 and IL-26. *Immunology Letters* 88:171-174.
- Cruz-Guilloty F, Pipkin ME, Djuretic IM, et al., 2009. Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. *The Journal of Experimental Medicine* 206:51-59.
- Dinau FC, González-Zambrano CM, Jimenez JJ, et al., 2025. Exploring the dynamics of IL-6, TGF- $\beta$ 1, and CD8<sup>+</sup> T cells in the canine transmissible venereal tumor: new perspectives. *Pakistan Veterinary Journal* 45(1): 375-381.
- Du Y, Wang K, Zi X, et al., 2025. Combined transcriptome and metabolome analysis of stable knockdown and overexpression of the CD8A gene in chicken T lymphocytes. *Poultry Science* 104:104686.
- Du Y, Zhang R, Li M, et al., 2026. Whole-transcriptome sequencing revealed the ceRNA regulatory network of stable knockdown of the CD8A gene in chicken T lymphocytes. *Poultry Science* 105:106318.
- Duan T, Du Y, Xing C, et al., 2022. Toll-like receptor signaling and its role in cell-mediated immunity. *Frontiers in Immunology* 13:812774.
- Gezer A, Baygütaalp NK, Cengiz M, et al., 2024. Cilomilast, a PDE4 inhibitor, suppresses CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation in the thymus and spleen of rats: mechanism of glutathione reduction. *Pakistan Veterinary Journal* 44(3): 707-714.
- Ghaffar A, 2025. Molecular prevalence, genetic diversity and in silico structural analysis of MRSA isolated from eggshell in commercial layer farms. *Pakistan Journal of Veterinary and Animal Research* 1(2): 39-45.
- Guo Y, Su A, Tian H, et al., 2020. Transcriptomic analysis of spleen revealed mechanism of dexamethasone-induced immune suppression in chicks. *Genes (Basel)* 11:513.
- Hua X, Hu G, Hu Q, et al., 2020. Single-cell RNA sequencing to dissect the immunological network of autoimmune myocarditis. *Circulation* 142:384-400.
- Huang Y, Urban C, Hubel P, et al., 2024. Protein turnover regulation is critical for influenza A virus infection. *Cell systems* 15:911-929.e8.
- Jia Y, Wang H, Ma B, et al., 2024. Lipid metabolism-related genes are involved in the occurrence of asthma and regulate the immune microenvironment. *BMC Genomics* 25:129.
- Jivanjee T, Ibrahim S, Nyquist SK, et al., 2022. Enriching and characterizing T cell repertoires from 3' barcoded single-cell whole transcriptome amplification products. *Methods of Molecular Biology* 2574:159-182.
- Lei M, Siemers NO, Pandya D, et al., 2021. Analyses of PD-L1 and inflammatory gene expression association with efficacy of Nivolumab $\pm$ Ipilimumab in gastric cancer/gastroesophageal junction cancer. *Clinical Cancer Research* 27:3926-3935.
- Leishman AJ, Naidenko OV, Attinger A, et al., 2001. T cell responses modulated through interaction between CD8 $\alpha$  and the nonclassical MHC class I molecule, TL. *Science* 294:1936-1939.
- Li R, Li D, Xu S, et al., 2024. Whole-transcriptome sequencing reveals a melanin-related ceRNA regulatory network in the breast muscle of Xichuan black-bone chicken. *Poultry Science* 103:103539.
- Ma M, Cai B, Jiang L, et al., 2018. lncRNA-Six1 is a target of miR-1611 that functions as a ceRNA to regulate Six1 protein expression and fiber type switching in chicken myogenesis. *Cells* 7:243.
- Manaiig YJY, Criado-Mesas L, Esteve-Codina A, et al., 2023. Identifying miRNA-mRNA regulatory networks on extreme n-6/n-3 polyunsaturated fatty acid ratio expression profiles in porcine skeletal muscle. *PLoS One* 18:e0282321.
- Mazhar F, Geng Q, Zhao Z, et al., 2025. Chicken primordial germ cells (pgcs) formation, regulation, and fate determination under omics approaches: a comprehensive scientific review. *Pakistan Veterinary Journal* 45(2): 475-487.
- Pearce EL, Mullen AC, Martins GA, et al., 2003. Control of effector CD8<sup>+</sup> T cell function by the transcription factor Eomesodermin. *Science* 302:1041-1043.
- Ribatti D, Tamma R, Komi DEA, 2019. The morphological basis of the development of the chick embryo immune system. *Experimental Cell Research* 381:323-329.
- Sallusto F, Lenig D, Förster R, et al., 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.
- Sang W, Sun C, Zhang C, et al., 2016. MicroRNA-150 negatively regulates the function of CD4<sup>+</sup> T cells through AKT3/Bim signaling pathway. *Cell Immunology* 306-307:35-40.
- Schmidt ME and Varga SM, 2018. The CD8 T cell response to respiratory virus infections. *Frontiers in Immunology* 9:678.
- Shang C, Li Y, Wu Z, et al., 2021. The prognostic value of DNA methylation, post-translational modifications and correlated with immune infiltrates in gynecologic cancers. *Pharmacogenomics and Personalized Medicine* 14:39-53.
- Shao L, Ye Q, Jia M, 2021. miR-130-3p Promotes MTX-Induced Immune Killing of Hepatocellular Carcinoma Cells by Targeting EPHB4. *Journal of Healthcare Engineering* 2021:4650794.
- Sohn JI, Nam K, Hong H, et al., 2018. Whole genome and transcriptome maps of the entirely black native Korean chicken breed Yeonsan Ogye. *Gigascience* 7:giy086.
- Suetake H, Araki K, Akatsu K, et al., 2007. Genomic organization and expression of CD8 $\alpha$  and CD8 $\beta$  genes in fugu *Takifugu rubripes*. *Fish Shellfish Immunology* 23:1107-1118.
- Takahama Y, Ohigashi I, Baik S, et al., 2017. Generation of diversity in thymic epithelial cells. *Nature reviews. Immunology* 17:295-305.
- Tang HL and Cyster JG, 1999. Chemokine Up-regulation and activated T cell attraction by maturing dendritic cells. *Science* 284:819-822.
- Vauthier V, Lasserre A, Morel M, et al., 2022. HUSH-mediated HIV silencing is independent of TASOR phosphorylation on threonine 819. *Retrovirology* 19:23.
- Vergadi E, Ieronymaki E, Lyroni K, et al., 2017. Akt signaling pathway in macrophage activation and M1/M2 polarization. *Journal of Immunology* 198:1006-1014.
- Vermeer BJ, Santerse B, Van De Kerckhove BA, et al., 1988. Differential immune response of congenic mice to ultraviolet-treated major histocompatibility complex class II-incompatible skin grafts. *Transplantation* 45:607-610.
- Wik JA and Skålhegg BS, 2022. T Cell Metabolism in Infection. *Frontiers in Immunology* 13:840610.
- Xiao C, Sun T, Yang Z, et al., 2022. Whole-transcriptome RNA sequencing reveals the global molecular responses and circRNA/lncRNA-miRNA-mRNA ceRNA regulatory network in chicken fat deposition. *Poultry Science* 101:102121.
- Xie T, Du Y, Wang Q, et al., 2026. A unique CD4<sup>+</sup> T cell subset expressing granzyme K is regulated by transcription factor EOMES and important for T cell-mediated intestinal inflammation. *Nature Immunology* 27:1212-1223.
- Xu Q, Chen Y, Zhao, WM, et al., 2014. DNA methylation and regulation of the CD8A after duck hepatitis virus type I infection. *PLoS One* 9:e88023.
- Yan C, Zhong Q, Jin W, et al., 2024. Epidemiological study of the co-infection of immunosuppressive poultry pathogens in tissue samples of chickens in Jiangsu Province, China from 2016 to 2022. *Pakistan Veterinary Journal* 44(2): 330-335.
- Zander R, Schauder D, Xin G, et al., 2019. CD4<sup>+</sup> T Cell help is required for the formation of a cytolytic CD8<sup>+</sup> T cell subset that protects against chronic infection and cancer. *Immunity* 51:1028-1042.e4.
- Zdrojewicz Z, Pachura E and Pachura P, 2016. The thymus: a forgotten, but very important organ. *Advances in Clinical and Experimental Medicine* 25:369-375.
- Zhai M, Guo Y, Su A, et al., 2020. Identification of genes related to dexamethasone-induced immunosuppression in chicken thymus using transcriptome analysis. *Research in Veterinary Science* 132:318-327.
- Zhang Q, Yang K, Huang Y, et al., 2017. Distribution of T-cell markers CD4 and CD8 $\alpha$  in lymphoid organs of healthy newborn, juvenile, and adult highland-plateau yaks. *American Journal of Veterinary Research* 78:609-617.
- Zheng L, Han X, Yao S, et al., 2022. The CD8 $\alpha$ -PILR $\alpha$  interaction maintains CD8<sup>+</sup> T cell quiescence. *Science* 376:996-1001.
- Zheng W, Su H, Lv X, et al., 2022. Exon-Intron circular RNA circRNF217 promotes innate immunity and antibacterial activity in teleost fish by reducing miR-130-3p function. *Journal of Immunology* 208:1099-1114.