Transcriptome alterations in chicken HD11 cells with steady knockdown and overexpression of \textit{RIPK2} gene

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ABSTRACT Receptor interacting protein kinase 2 (RIPK2) is involved in a variety of signaling pathway to produce a series of inflammatory cytokines in response to a diverse of bacterial, viral and protozoal pathogens. However, the underlying regulating of RIPK2 remain unknown. Transcriptome alterations in chicken HD11 cells following RIPK2 overexpression or silencing by shRNA were analyzed by next-generation sequencing. Both overexpression and knockdown of the RIPK2 gene caused wide-spread changes in gene expression in chicken HD11 cells. Differentially expressed genes (DEGs) caused by altered RIPK2 gene expression were associated with multiple biological processes linked with biological regulation, response to stimulus, cell communication, and signal transduction etc. KEGG analysis revealed that many of the DEGs were enriched in VEGF signaling pathway, ECM-receptor interaction, Focal adhesion, TGF-beta signaling pathway etc. Moreover, we show that initiation genes, TGFB1 and TGFB3, in the TGF-beta signaling pathway are biological targets regulated by RIPK2 in chicken HD11 cells. This is the first transcriptome-wide study in which RIPK2-regulated genes in chicken cells have been screened. Our findings elucidate the molecular events associated with RIPK2 in chicken HD11 cells.

Key words: RIPK2, knockdown, overexpression, RNAseq, gene network

INTRODUCTION

Receptor interacting protein kinase 2 (RIPK2), a member of the RIPK family, is a critical effector of immunity to a diversity of bacterial, viral, and protozoal pathogens as it is an essential adaptor for a variety of signal transduction cascades. Specifically, RIPK2 could participate in innate and acquired immune responses induced by different pathogens to produce a series of inflammatory cytokines and regulate cell apoptosis (Bist et al., 2014; Hofmann et al., 2021). Previous studies have shown that RIPK2 mainly exhibited its function through NOD signaling pathway, which is a key and core gene of NOD signaling pathway and a necessary gene to activate ERK and MAPK pathways (Jing et al., 2014; Li et al., 2019). Activation the complex of NOD2: RIPK2 can cause a series of cascade reactions such as autophagy, antigen presentation, and initiation of a series of signaling pathways (Anand et al., 2011; Wu et al., 2018; Lin et al., 2020; Pan et al., 2021; Zhou et al., 2021).

Moreover, RIPK2 can also participate in many diseases such as pneumonia, multiple sclerosis, asthma, Crohn’s disease (Balamayooran et al., 2011; Shaw et al., 2011; Miller et al., 2020), playing an important role in respiratory system immunity. Shimada et al. (2009) have demonstrated that knockout of RIPK2 in mice could impair iNOS expression and NO production; delay neutrophil recruitment to the lungs; reduce bacterial clearance; and develop more severe and chronic lung inflammation. Meanwhile, it was found that RIPK2 played a critical role in the activation of central nervous system-infiltrating dendritic cells, as well as in autoimmune encephalomyelitis (Shaw et al., 2011). Besides, Miller et al. (2020) found that RIPK2 can promote house dust mite-associated allergic airway inflammation and Th2 and Th17 immunity. Inhibition of RIPK2 can attenuate airway inflammation and remodel airway (Miller et al., 2020), indicating RIPK2 might be a potential of pharmacological targeting gene in asthma.
The aforementioned findings reveal that RIPK2 plays an important role in the recognition of intracellular bacteria, the alleviation of inflammatory responses in different diseases, and the regulation of host immunity. At present, although the function of RIPK2 gene and its NOD signaling pathway have been studied in human and mice, there are few studies in chickens. Furthermore, the gene networks regulating RIPK2 are unclear at present.

In the present study, we performed a large-scale analysis of gene expression alterations in chicken HD11 cells induced by RIPK2 knockdown and overexpression using transcriptome sequencing. Furthermore, we identified the genes involved in the TGF-beta signaling pathway as targets of RIPK2 regulation in chicken HD11 cells. These results have provided new insights into the role of RIPK2 in chicken.

**MATERIALS AND METHODS**

**Ethics Statement and Experimental Animals**

The procedures involving animals and their care conformed to the U.S. National Institute of Health guidelines (NIH Pub. No. 85-23, revised 1996). The experiments were conducted under the approval of the Ethics Committee of Yangzhou University for Laboratory and Experimental Animals. The chickens used in this study were obtained from the TIANGE QINYE Co., Ltd. The experimental procedures were approved by the Institutional Animal Care and Use.

**Experiment and Data Acquisition**

The RNA-seq data of bone marrow were from our previous experiment, which can be downloaded from Gene Expression Omnibus (GEO) with accession numbers GSE67302. The detailed information of bone marrow RNA-seq experiment can be found in the study of Sun et al. (2015). Briefly, 4 wk commercial male broilers were challenged with 0.1 mL 1 × 10^8 cfu APEC O1 via the left intra-air sac injection. The control birds were injected with 0.1 mL PBS with the same route. Bone marrow was collected at 5 days postinfection (dpi). The infected birds were categorized into resistant and susceptible based on internal lesion scores according to the study of Peighambari et al. (2000). The lesion score of the resistant birds was 0–1, while that of susceptible birds were 6–7. Then, a total of 3 treatments were generated: control birds, resistant birds, and susceptible birds.

**Analysis of RIPK2 Gene Expression Patterns**

A total of 13 tissues (heart, cecum, stomach, liver, small intestine, duodenum, lung, harderian glands, bone marrow, bursa, thymus, spleen, and blood) were collected respectively from four 4 wk of age commercial male broilers. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) to identify the RIPK2 gene expression pattern by using RT-qPCR.

**Cell Culture**

The chicken HD11 cell line was kindly provided by Dr. Xuming Hu (Yangzhou University). The chicken macrophage-like cell line HD11 was maintained in RPMI1640 (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gibco) in a humidified incubator with 5% CO_2 at 37°C, and cells were passaged before 80 to 90% confluence.

**Cell Transfection and Infection**

Small hairpin RIPK2 (shRIPK2) plasmid (Table S1) and pcDNA3.1-RIPK2 (oeRIPK2) were synthesized by GenePharma (Shanghai, China). The recombinant vector (shRIPK2 with high interference efficiency and oeRIPK2) were packaged with lentivirus. The lentivirus titer is 2×10^8 TU/mL. 1×10^5 cells/well were seeded in the 24 well plate. The cells were then infected with lentivirus with MOI = 40 by using the lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After 48 h of cell culture in 37°C with 5% CO_2 for shRIPK2 and oeRIPK2 group, 10 μg/mL purinomycin medium was added in the cells. Then, the positive clones were screened for four generations, and finally the HD11 cells with steady overexpression/knockdown of RIPK2 gene was obtained. For induction with LPS, HD11 cells with steady knockdown or overexpression of RIPK2 were challenged with 1 μg/mL LPS for 24 h.

**Apoptosis Assay**

Cell apoptosis was evaluated using an annexin V-PE/7-AAD apoptosis detection Kit (Vazyme, Nanjing, China). In brief, cells (5 × 10^7 cells/well) were divided into different groups and seeded in 6-well plates. After transfection, the cells were placed in 500 μL of a binding buffer (Biosea Biotechnology, Beijing, China), treated using 5 μL of annexin V-PE and 10 μL of 7-AAD, main-ained for 30 min at 25°C in the dark. Stained cells were detected and analyzed using flow cytometry (Becton Dickinson, Franklin Lakes, NJ). Each experiment was performed in triplicate.

**Transcriptome Sequencing and Gene Expression Quantitation**

Total RNA was extracted from HD11 cells of wild type group (WT), knockdown of RIPK2 group (shRIPK2), and overexpression of RIPK2 group (oeRIPK2) using an RNA isolation kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. The quality of RNA was analyzed by agarose gel electrophoresis and a Nanodrop OneCspectrophotometer (Thermo Fisher Scientific Inc., MA). The RNA Integrity was confirmed by Qseq (Qseq100, Guangding, Taiwan). A total of 2 μg of RNA...
was used for stranded RNA sequencing library preparation using Ribo-of rRNA depletion kit (Catalog NO. MRZG12324, Illumina, San Diego, CA) following the manufacturer’s instruction. The library products corresponding to 200 to 500 bps were enriched, quantified, and finally sequenced on NovaSeq 6000 sequencer (Illumina) with PE150 sequencing platform.

For quality control, raw data were filtered by Trimmomatic (version 0.36). The obtained clean reads were further treated with in-house scripts to eliminate duplicate bias introduced in library preparation and sequencing. Duplicated reads were mapped to the reference genome of the chicken (*Gallus gallus*) from Ensembl ([https://asia.ensembl.org/info/data/ftp/index.html](https://asia.ensembl.org/info/data/ftp/index.html)) using STRA software (version 2.5.3a) with default parameters. Reads mapped to the exon regions of each gene were counted by featureCounts (Subread-1.5.1; Bioconductor) and then RPKM was calculated. Differentially expressed genes (DEGs) between groups were identified using the edgeR package (version 3.12.1). A *P*-value cutoff of 0.05 and fold-change cutoff of 1.5 were used to judge the statistical significance of gene expression differences.

**Functional Categorization**

GO analysis ([Huang et al., 2009a](#)) and KEGG enrichment analysis ([Kanehisa and Goto, 2000; Huang et al., 2009b](#)) for DEGs were both implemented by KOBAS software (version: 2.1.1) with a *P*-value cutoff of 0.05 to judge statistically significant enrichment.

**Quantitative Real-Time PCR Assay**

The RNA from different experiments was reverse transcribed into cDNA using a Reverse Transcription Kit (Takara, Dalian, China). The One Step SYBR Premix Ex Taq II kit (Takara) to evaluate the expression level of GAPDH, RIPK2, HSPB1, JUN, FOS, CASP3, BCL2L11, CD44, CD36, TNFSF8, PDK4, IRF1, PAK3, and CXCR1. Primer sequences are displayed in Table S2. RT-qPCR thermal cycling conditions were as follows: denaturation for 3 min at 95°C, 40 cycles of 10 s at 95°C, 58°C for 30 s, and then 72°C for 30 s. Relative expression of above genes were calculated using the 2^−ΔΔCt method and GAPDH was utilized as an internal control. The formula of ΔΔCt is (Ct of gene in test group - Ct of GAPDH in test group) - (Ct of gene in control group - Ct of GAPDH in control group).

**Western Blotting**

Cells from each of the 3 groups were lysed on ice using 200 µL RIPA bufer (Beyotime Biotec, Shanghai, China) for 30 min. Next, the lysis mixtures were centrifuged and the supernatants were collected. BCA Protein Assay Kit (Pierce, Appleton, WI) was used for quantification of proteins. Then, the isolated proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and electrophoretically transferred to PVDF membranes. Afterwards, membranes were blocked in 5% BSA for 2 h at room temperature and then probed with the primary antibodies at 4°C overnight. The primary antibodies included anti-GAPDH (ab181602, Abcam, Cambridge, MA), anti-RIPK2 (70R-10,459, Fitzgerald, North Acton, MA), anti-TGFβ1 (ab9758, Abcam), and anti-TGFβ3 (ab15537, Abcam) were used at a dilution of 1:1,000. Then the membranes were incubated with secondary antibodies tagged with horseradish peroxidases (Sigma-Aldrich, St. Louis, MI) at a 1:10,000 dilution at room temperature for 2 h. Then, immunoblots were visualized by enhanced chemiluminescence (ECL kit, Santa Cruz Biotechnology, Dallas, TX). The blots were visualized by using Image Lab Software (BioRad, Hercules, CA).

**Statistical Analysis**

All data were presented as mean ± standard error. Statistical analysis was performed using JMP statistical software (version 15.2.1, SAS Institute). Differential gene expression was analyzed using Student’s *t* test for 2 groups and one-way ANOVA with Tukey Honestly Significant differences test (HSD; SAS, 2000; Cary, NC). *P* < 0.05 was considered significant.

**RESULTS**

**Screening of RIPK2, a Key Candidate Gene for Host Against Bacteria Infection**

Bone marrow was collected from wild type birds (Non-infected APEC birds), susceptible birds (APEC-infected birds), and resistant birds (APEC-infected birds), and then performed by RNASeq ([Sun et al., 2015](#)). According to the results, 2,804, 1,873, and 2 differentially expressed genes (DEGs) were found in the comparison of Susceptible vs. Wild type birds, Susceptible vs. Resistant birds, and Resistant vs. Wild type birds, respectively. Then, transcriptome sequencing data were analyzed by Venn between Susceptible vs. Resistant birds and Susceptible vs. Wild type birds to find key candidate genes and pathways against APEC infection. A total of 1731 DEGs were identified and involved in 33 significantly changed pathways, of which NOD/RIPK2 signaling pathway had the smallest corrected *P* value ([Figure 1A](#)). The expression of DEGs involved in NOD/RIPK2 signaling pathway was displayed in Figure 1B. Since *RIPK2* was the core and key gene in NOD/RIPK2 signaling pathway and had a high expression level in the comparison of Susceptible vs. Resistant birds at 5 d post APEC infection, we speculate that it may play a critical role in host against bacterial infection.
Expression Level of RIPK2 in Different Tissues

Total RNA was extracted from 13 tissues (heart, cecum, stomach, liver, small intestine, duodenum, lung, harderian glands, bone marrow, bursa, thymus, spleen, and blood). RT-qPCR was used to analyze the mRNA expression levels of RIPK2 in different tissues. Compared with the heart, the relative RIPK2 expression level in other tissues was measured as shown in Figure 2. The mRNA expression level of RIPK2 gene was highest in the bone marrow ($P < 0.01$) compared to other tissues, followed by spleen, blood, thymus, bursa, lung, stomach, harderian glands, small intestine, and cecum. However, the liver tissue had the smallest mRNA expression level of
RIPK2 gene compared with heart tissues, followed by duodenum.

**Construction of Overexpression and Interference Lentiviral Vector of RIPK2**

To determine the target genes of RIPK2, the lentivirus interference and overexpression vector of RIPK2 were designed and constructed (Figure 3A). The constructed vector with GFP fluorescent protein labeling was transfected into chicken HD11 cells. A large number of fluorescent expressions was observed after 48 h transfection with the constructed vector (Figures 3B and 3C), indicating the vectors can be successfully transfected and effectively expressed in vitro. Moreover, after interfering with RIPK2, the interference vector 1, 2, and 3 could significantly reduce the mRNA expression level of RIPK2 gene \((P < 0.01)\), of which interference vector 3 can strongest affect the RIPK2 expression and was selected to package lentivirus for follow-up experiments (Figure 3B). Meanwhile, the overexpression vector can significantly increase the mRNA expression level of RIPK2 gene (Figure 3C). In summary, the recombinant vector could effectively interfere/overexpress RIPK2 in chicken HD11 cells in vitro.

**Expression Level of RIPK2 in HD11 Cells With Steady Knockdown and Oveexpression of RIPK2**

After 48 h transfection with 20 \(\mu\)L of lentivirus encapsulated shRIPK2-3 vector, chicken HD11 cells were cultured with 10 \(\mu\)g/mL puromycin and incubated four generation to obtain the steady RIPK2 knockdown HD11 cells. The steady RIPK2 overexpression HD11 cells were also obtained with the same method. To identify the activity of RIPK2, the protein and mRNA levels were measured in wild type HD11 cells (WT), HD11 cells with knockdown of RIPK2 (shRIPK2), and HD11 cells with overexpression of RIPK2 (oeRIPK2). As shown in Figure 4A and B, a significantly decreased mRNA and protein expression level of RIPK2 were observed in the RIPK2 knockdown group in comparison to WT \((P < 0.05)\), indicating the expression of RIPK2 was indeed inhibited. Also, the oeRIPK2 group can significantly increase mRNA and protein abundance of
RIPK2 compared with WT ($P < 0.05$). In summary, we had successfully constructed the steady RIPK2 knockdown and overexpression HD11 cells.

**Knockdown of RIPK2 Alleviate Cell Injuries and Overexpression of RIPK2 Enhanced Cell Injuries in LPS-Induced Cells**

To identify the function of RIPK2 during bacterial infection, the effects of RIPK2 on LPS-induced injuries in chicken HD11 cells were analyzed. Overexpression of RIPK2 can aggravate LPS-induced injuries in cells by decreasing viability (Figures 5A and 5B), increasing apoptosis (Figures 5A and 5C). However, inhibition of RIPK2 exhibited the contrary effects on chicken HD11 cells. The viability was improved (Figures 5A and 5B), and apoptosis was inhibited (Figures 5A and 5C). These data suggested that knockdown of RIPK2 can alleviate inflammatory injuries in chicken HD11 cells.

**RNA-Seq and Data Analysis**

A total of nine cDNA libraries were constructed, respectively, from WT ($n = 3$), shRIPK2 ($n = 3$), and oeRIPK2 ($n = 3$). The quality control of sequencing data is shown in Table 1. In general, an average of 79,839,400 (range from 71,438,338 to 89,995,934) raw reads was obtained (Table 1). After removing adaptors and low quality reads, each sample yielded approximately 77,545,511 clean reads (range from 69,393,540 to 87,645,300, Table 1). Moreover, unique identifier (UID) was also used to remove the duplication data, resulting in a total of 48,275,868 to 60,443,170 UID reads (Table 1). The average of GC content of clean reads was 54.27% (Table 1).

Moreover, 92.81 to 94.83% of the UID reads were found to successfully map to the chicken reference genome (*Gallus gallus* 6.0), of which 86.14 to 91.25% were uniquely mapped to genome (Table 2). Additionally, Table 3 shows the information on the fraction of reads mapping to features for each sample, that is, any expressed parts of the genome. Interestingly, oeRIPK2 group had the largest percentage of reads mapping to coding sequence (CDS, average percentage = 63.98%), followed by shRIPK2 (average percentage = 59.91%), and WT group (average percentage = 59.78%). However, oeRIPK2 group had the smallest percentage of reads mapping to intron (average percentage = 17.84%) compared to shRIPK2 (average percentage = 19.19%) and WT group (average percentage = 19.41%).

![Figure 4](image-url). The RIPK2 expression level in chicken HD11 cells with knockdown or overexpression of RIPK2. (A) The relative RIPK2 mRNA expression level in wild type HD11 cells (WT), knockdown of RIPK2 HD11 cells (shRIPK2), and overexpression of RIPK2 HD11 cells (oeRIPK2). (B) The RIPK2 protein expression level in wild type HD11 cells (WT), knockdown of RIPK2 HD11 cells (shRIPK2), and overexpression of RIPK2 HD11 cells (oeRIPK2) was analyzed by western blot. (C) Image J software was used for RIPK2 gray-level analysis of western blot results. ** $P < 0.01$. All values are mean±SD. $n = 4$. 

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Differentially Expressed Genes in HD11 Cells With Altered RIPK2 Expression Analyzed by Transcriptome Sequencing

To explore the gene network regulated by chicken RIPK2, we performed transcriptome sequencing using chicken HD11 cells with overexpression of RIPK2 gene (oeRIPK2) or with silenced RIPK2 gene expression using shRNA (shRIPK2), and wild type HD11 cells (WT). As shown in Figure 6A, the gene expression pattern of WT group was more similar with that in shRIPK2 group, whereas the expression pattern of oeRIPK2 group was...
completely different from WT and shRIPK2 group. Compared with WT group, 1,472 and 1,133 DEGs were identified to be upregulated and downregulated, respectively, in shRIPK2 group (Figure 6B), while 5,053 and 5,416 genes was significantly high expressed and low expressed, respectively, in oeRIPK2 group (Figure 6C). It was found that 4,881 and 5,416 were significantly upregulated and downregulated, respectively, in the comparison of oeRIPK2 vs. shRIPK2 (Figure 6D). The distribution of log$_2$(fold change) in shRIPK2 vs. WT was ranged from $-3.5$ to 3.5, whereas those in oeRIPK2 vs. WT and oeRIPK2 vs. shRIPK2 were from $-12$ to 12 (Figure 6E). Moreover, 1,670 overlapped DEGs were identified in the three comparisons (Figure 6F).

## Functional Annotation of DEGs in Different Comparisons

The gene ontology (GO) classification system was used to classify the possible functions of DEGs in different comparisons. A total of 734 genes (28.18%), 1,476 (31.46%), and 3,193 (31%) were successfully assigned to at least one GO term annotation in shRIPK2 vs. WT, oeRIPK2 vs. WT, and oeRIPK2 vs. shRIPK2, respectively. The GO enrichment analysis of DEGs in shRIPK2 vs. WT showed that a total of 404 terms were enriched, including regulation of cellular process, regulation of biological process, biological regulation, and binding etc (Figure 7A). A total of 1,010 significant GO terms were identified in the comparison of oeRIPK2 vs. shRIPK2, of which the top 20 GO terms were similar with shRIPK2 vs. WT (Table S3), oeRIPK2 vs. WT (Table S4), and oeRIPK2 vs. shRIPK2 (Table S5), respectively. Moreover, 1,042 GO terms were preferentially enriched in cellular process, regulation of biological process, response to stimulus, gene expression etc. (Figure 7B).

Then, Kyoto Encyclopedia of Genes and Genomes (KEGG) classification system was also performed to identify the possible functions of DEGs. A total of 15, 85, and 86 significantly changed pathways were identified in shRIPK2 vs. WT (Table S3), oeRIPK2 vs. WT (Table S4), and oeRIPK2 vs. shRIPK2 (Table S5), respectively. Moreover, A total of 11 significantly changed pathways were detected to be overlapped in the 3 comparisons, which mainly included “Herpes simplex infection”, “Focal adhesion”, “MAPK signaling pathway”, “ECM-receptor interaction”, “Lysoosome”, “VEGF signaling pathway”, “Phagosome”, and “Apoptosis” etc. (Figure 7D). More remarkable, the significantly changed pathway “Notch signaling pathway (cell growth and differentiation, cell fate decisions)”, “TGF-beta signaling pathway (cell proliferation, apoptosis, differentiation, and migration)”, “Cell cycle (cell division)”, and “Oxidative phosphorylation (cellular respiration)” were enriched in both oeRIPK2 vs. WT and oeRIPK2 vs. shRIPK2.

## Verification of RNAseq Data by RT-qPCR

To validate the reliability of RNA-seq data, 12 DEGs overlapped in the 3 comparisons were randomly selected...
for relative expression analysis by RT-qPCR (Figure 8). The results showed that the relative expression of JUN, FOS, BCL2L11, CD44, CD36, TNFSF8, PDK4, IRF1, and CXCR1 in oeRIPK2 group were significantly lower than those in WT and shRIPK2 group (P < 0.01), whereas the relative expression of HSPB1, CASP3, and PAK3 in oeRIPK2 group were significantly higher than those in WT and shRIPK2 group. The expression tendency (upregulated or downregulated) of the aforementioned DEGs in the comparison of shRIPK2 vs. WT was completely opposite to those in oeRIPK2 vs. WT and oeRIPK2 vs. shRIPK2. The results of RT-qPCR validation were consistent with RNA-seq data, which confirms the reliability of the RNA-seq results.

RIPK2 is a Critical Regulator for TGF-Beta Signaling Pathway

In the current study, TGF-beta signaling pathway was significantly activated with RIPK2 overexpression or silenced. A total of 34 and 28 upregulated DEGs were involved in TGF-beta signaling pathway in oeRIPK2 vs. WT and oeRIPK2 vs. shRIPK2, respectively, as shown in Tables S6 and S7. 46 DEGs were commonly involved in the TGF-beta signaling pathway in both oeRIPK2 vs. WT and oeRIPK2 vs. shRIPK2 (Figure 9A). The fold change of the overlapped DEGs ranged from −49.15 to 89.23 and from −39.44 to 82.18 in the comparison of oeRIPK2 vs. WT and oeRIPK2 vs. shRIPK2, respectively (Tables S6 and 7). To further corroborate the correlation between RIPK2 and TGF-beta signaling pathway, we used RT-qPCR and western blotting to identify the expression level of TGFB1 and TGFB3 with the RIPK2 gene overexpression or silenced. As shown in Figures 9B–9F, the mRNA and protein levels of TGFB1 or TGFB3 showed remarkable increase in the comparison of oeRIPK2 vs. WT, which is in agreement with the RNA-seq data. However, compared to the WT group, the mRNA and protein expression level of TGFB1 or TGFB3 were significantly decreased in the shRIPK2 group (Figures 9B–9F). These results indicate RIPK2 is involved in the regulation of TGFB1 and TGFB3 in a certain relationship to further modulate the TGF-beta signaling pathway.

DISCUSSION

Receptor interacting protein kinase (RIPK) family has 5 important members in chicken, including RIPK1, 2, 3, 4, and 5. However, there were 8 members (RIPK1, 2, 3, 4, 5, 6, 7, and 8) of RIPK in human. Currently, among the members of RIPK family, researchers mainly focused on the RIPK2 gene, since it is the key and core gene in NOD signaling pathway. RIPK2 plays an important role in protecting the body from a variety of stimuli and pathological conditions (Zhao et al., 2017; Pham et al., 2020; Honjo et al., 2021). Although there have been many studies on RIPK2 in humans and mice,
its regulatory gene network in poultry has not been carried out. This study is the first to demonstrate the essential genes or pathways, as well as the downstream targets regulated by RIPK2 through transcriptome analysis.

Chicken RIPK2 is located on chromosome 2 and contains 12 exons. In the current study, the expression level of RIPK2 gene in 13 tissues was analyzed. It was found that chicken bone marrow had the highest RIPK2 expression level, which is consistent with the previous findings in human (Fagerberg et al., 2014). However, the expression level of RIPK2 in liver was different between human and chicken (Fagerberg et al., 2014). Herein, we found that inhibition of chicken RIPK2 can attenuate the LPS-induced cell injuries, which is consistent with the findings in human and mice. To further identify the gene networks or pathways regulated by RIPK2, we compared the transcriptomes of each 2 groups (WT, shRIPK2, and oeRIPK2) to find the significantly changed genes and pathways. It was found that Focal adhesion, MAPK signaling pathway, ECM-receptor interaction, Lysosome, and Apoptosis were commonly significantly changed in the three comparisons (shRIPK2 vs. WT, oeRIPK2 vs. WT, and oeRIPK2 vs. shRIPK2). Yang et al. demonstrated that knockdown of RIPK2 could inhibit the apoptosis pathway in human gastric cancer (Yang et al., 2021). Moreover, Pan et al. and Cai et al. have separately reported that silencing of RIPK2 suppressed the MAPK signaling pathway (Cai et al., 2018; Pan et al., 2021). The aforementioned research findings were in agreement with current results. Furthermore, we found Focal adhesion, ECM-receptor interaction, Lysosome, VEGF signaling pathway, and Phagosome were

**Figure 7.** Gene classification was based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis for differentially expressed genes (DEGs). (A−C) Different classes are shown for biological processes, cellular components, and molecular functions in the comparisons of RIPK2 knockdown HD11 cells (shRIPK2) vs. wild type HD11 cells (WT) (A), RIPK2 overexpression HD11 cells vs. WT (B), and oeRIPK2 vs. shRIPK2 (C). (D) Overlap in significantly changed pathways for the comparisons of shRIPK2 vs. WT, oeRIPK2 vs. WT, and oeRIPK2 vs. shRIPK2.
closely correlated with RIPK2 regulation. These results were reasonable and expected, since RIPK2 was involved in cellular immune response (Usluoglu et al., 2007; Sandford et al., 2011; Sun et al., 2015, 2016) and knockdown of RIPK2 could avoid the excessive tissues/cells injury and inflammatory response (Wu et al., 2012; Rahman et al., 2014; Yang et al., 2021).

Moreover, it was worth to note that the TGF-beta signaling pathway was also detected to be significantly changed in the comparison of oeRIPK2 vs. WT and oeRIPK2 vs. shRIPK2. The TGF-beta signaling pathway plays an important role in the development, homeostasis, and repair of most tissues in organisms (Kitisin et al., 2007; Chen et al., 2012; Tzavlaki and Moustakas, 2020). It has been demonstrated that TGF-beta signaling pathway presents broad therapeutic potential in many diseases, including various cancers, hepatic fibrosis, inflammatory bowel disease etc. (Xu et al., 2016; Colak and Ten Dijke, 2017; Ihara et al., 2017). Since TGBF1 and TGBF3 were the initiation genes of chicken TGF-beta signaling pathway, we subsequently validated the relationship between RIPK2 and TGFB1/TGBF3. Our transcriptome data showed that TGBF1 and TGBF3 were significantly increased in the comparisons of oeRIPK2 vs. WT (Fold change of TGBF1 = 3.14; Fold change of TGBF3 = 8.04) and oeRIPK2 vs. shRIPK2 (Fold change of TGBF1 = 2.45; Fold change of TGBF3 = 10.75). Moreover, we validated these results finding decreased expression levels of both mRNA and protein for TGFB1 and TGFB3 in shRIPK2 group, compared to WT. However, data obtained by RT-qPCR and western blot showed the expression of TGFB1 and TGFB3 were significantly upregulated in the comparison of oeRIPK2 vs. shRIPK2. These results strongly suggested TGFB1 and TGFB3 were the downstream targets of RIPK2 and positively modulated by RIPK2 in chicken HD11 cells.

It has been demonstrated that the TGFB1 has the function to regulate cell proliferation, differentiation and growth, and can modulate expression and activation of other growth factors including interferon gamma and tumor necrosis factor alpha (Lafontaine et al., 2011; Ma et al., 2019; Moraveji et al., 2019). TGFB3 is involved in embryogenesis and cell differentiation, and may play a role in wound healing (Lichtman et al., 2016; Stockis et al., 2017; Tamayo et al., 2018). It has been

![Figure 8. Evaluation and comparison of mRNA expression levels of the selected differentially expressed genes (DEGs) by using RNA-seq and RT-qPCR for different comparisons. (A–C) The mRNA expression level of the differentially expressed genes in the comparisons of RIPK2 knockdown HD11 cells (shRIPK2) vs. wild type HD11 cells (WT) (A), RIPK2 overexpression HD11 cells (oeRIPK2) vs. WT (B), and oeRIPK2 vs. shRIPK2 (C).](image-url)
reported that mutation of \textit{TGFB3} is a cause of aortic aneurysms and dissections, as well as familial arrhythmogenic right ventricular dysplasia 1 (Beffagna et al., 2005; Bertoli-Avella et al., 2015). According to our results, \textit{RIPK2} has the potential ability to involve in cell proliferation, differentiation, growth, and different disease via regulating the TGF-beta signaling pathway.

\section*{CONCLUSIONS}

In summary, this study has provided an analysis of the genetic landscape associated with \textit{RIPK2} knockdown or overexpression in chicken HD11 macrophages. In total, 2,605, 10,469, and 10,297 DEGs were identified in shRIPK2 vs. WT, oeRIPK2 vs. WT, and oeRIPK2 vs. shRIPK2, respectively. Functional annotation analysis showed that apoptosis, MAPK, p53, ECM-receptor interaction, Lysosome, VEGF signaling pathway, Phagosome, and TGF-beta signaling pathway were involved in \textit{RIPK2} knockdown/overexpression HD11 cells. By analyzing the enriched pathway and gene networks, we identified that TGF-beta signaling pathway, especially the initiation genes \textit{TGFB1} and \textit{TGFB3}, were targeted by \textit{RIPK2} gene. As a whole, this study can not only provide data support for constructing gene networks related to \textit{RIPK2}, but also provide new ideas for the functions of \textit{RIPK2}.

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\section*{DISCLOSURES}

The authors declare that they have no conflict of interest.

\section*{SUPPLEMENTARY MATERIALS}

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