# Profiling and analysis of exosomal miRNAs derived from highly pathogenic avian influenza virus H5N1-infected White Leghorn chickens

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**ABSTRACT** Exosomes are small cell membrane-derived vesicles; they play important roles as mediators of cell-to-cell communication via delivery of their contents, such as proteins and microRNAs (**miRNAs**). In particular, exosomal miRNAs regulate the gene expression of recipient cells by inhibiting the expression of target mRNAs. In this study, we investigated the miRNA expression profiles of highly pathogenic avian influenza virus (**HPAIV**) H5N1-infected White Leghorn chickens and analyzed the functions of their target genes. After 3 d of infection with A/chicken/Vietnam/NA-01/2019 (H5N1), exosomes were isolated from the blood serum of White Leghorn chickens for small RNA sequencing. We accordingly identified 10 differentially expressed miRNAs (**DE miRNAs**; 5 upregulated and 5 downregulated) by comparing the exosomes derived from infected and noninfected chickens. The target genes of DE miRNAs were predicted using miRDB and TargetScan for Gene Ontology and KEGG pathway enrichment analyses. A majority of the target genes was found to be associated with the MAPK signaling pathway; several immune-related genes were identified as being regulated by these DE miRNAs. Moreover, we predicted DE miRNA binding sites in HPAIV RNA segments using the RNAhybrid algorithm. The findings of this study provide a theoretical basis for gaining insights into the regulatory mechanisms of exosomal miRNAs in response to HPAIV H5N1 infection and the identification of novel vaccine candidates.

**Key words:** HPAIV, H5N1, exosome, miRNA, chicken

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## INTRODUCTION

Highly pathogenic avian influenza virus (**HPAIV**) H5N1, which was initially isolated in Guangdong, China, is associated with high lethality and morbidity, and has caused considerable damage to the poultry industry worldwide (Swayne and Suarez, 2000; Guan et al., 2004). Moreover, given that the H5N1 subtype can be transmitted to humans, causing more than 50% mortality, it poses a substantial threat to public health (Chen et al., 2021). However, owing to the poor cost-effectiveness of vaccinating poultry to control HPAIV H5N1, avian influenza continues to occur worldwide. Consequently, studies on the immune responses to H5N1 infection are considered necessary prerequisite for effectively controlling of the spread of HPAIV H5N1.

Exosomes are small (30−150 nm) membrane-derived vesicles found in biological fluids, including blood, urine, and milk (Zhang et al., 2019; Xie et al., 2020), which mediate cell-to-cell communication by delivering components such as DNA, RNA, proteins, and lipids from donor to recipient cells (Srikanthan et al., 2014; Boulanger et al., 2017). Notable among these exosomal components are microRNAs (**miRNAs**), which regulate gene expression in recipient cells by inhibiting the translation of target messenger RNAs (**mRNAs**) (Zhang et al., 2015; Alipoor et al., 2016; Chen et al., 2020).

It has been reported that exosomal miRNAs regulate immune responses against viral infection. For example, upregulation of exosomal miR-423-5p in response to rabies virus infection has been observed to enhance antiviral activity by inhibiting the expression of suppressor of cytokine signaling 3 (**SOCS3**) and inducing the production of interferon (**IFN**)-$\beta$ (Wang et al., 2019). Moreover, it has been found that upon infection with



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HPAIV H5N1, there is an increase in the expression of exosomal miR-483-3p in mouse serum, along with an induction of proinflammatory cytokine release (Maemura et al., 2020).

In our previous study, we reported the expression of exosomal miRNAs in response to H5N1 infection in Vietnamese native Ri chickens, among which 20 miRNAs were differentially expressed between infected and noninfected chickens (Hong et al., 2021). However, given that in many countries, White Leghorn chickens are among the most commonly bred commercial layers, and outbreaks of avian influenza virus in this breed incur serious economic losses; the profiling and analysis of exosomal miRNAs in these birds is thus considered a matter of priority. Moreover, considering the immunological importance of exosomes, it is necessary to study the exosomal miRNAs associated with the response to avian influenza virus infection for the development of effective avian influenza virus control methods, as well as for elucidating the mechanisms whereby these miRNAs respond to AIV infection. Accordingly, in this study, we performed miRNA profiling of H5N1-infected White Leghorn chickens and analyzed the predicted target genes.

## MATERIALS AND METHODS

### Experimental Birds and HPAIV Infection

Specific-pathogen-free (**SPF**) White Leghorn chickens were purchased from the Poultry Research Centre of the National Institute of Animal Science, Vietnam, and observed daily for signs of disease and mortality. All chicken infection experiments were performed in the biosafety level 2 plus (**BSL2**+) facility of our collaborative laboratory in the Department of Biochemistry and Immunology, National Institute of Veterinary Research, Vietnam. For the purposes of HPAIV challenge, 5 chickens (4-wk-old) were administered an intranasal inoculation of 100 $\mu$L of harvested allantoic fluid from infected eggs, containing a $1 \times 10^4$ 50% egg infectious dose (**$EID_{50}$**) of A/chicken/Vietnam/NA-01/2019 (H5N1), according to the World Organization for Animal Health (OIE) guidelines. Five noninfected chickens served as a control group. Experimental birds groups was housed separately in the isolation units. The protocol for virus infection was approved by the relevant committees at the National Institute of Veterinary Research (**NIVR**), Vietnam (TCVN 8402:2010/TCVN 8400-26:2014).

### Exosome Isolation and Characterization

Blood samples were collected from the wing vein of chickens (4 noninfected chickens and 4 infected chickens) after 3 d of infection. Exosomes from the noninfected and H5N1 AIV-infected chickens were isolated from the respective sera using Total Exosome Isolation Reagent (Invitrogen, Carlsbad, CA), following previously described methods (Hong et al., 2021). The size of the purified exosomes were measured using an SZ-100 nanoparticle analyzer (HORIBA, Kyoto, Japan). Western blotting was used to detect the exosomal markers CD9 (#13174, Cell Signaling Technology, Danvers, MA) and CD81 (#56039, Cell Signaling Technology).

### Exosomal RNA Extraction and Small RNA Sequencing

Exosomal RNA was extracted using miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Library construction and small RNA sequencing were conducted for exosomes extracted from both noninfected and H5N1 AIV-infected chickens. Briefly, the libraries were constructed using a SMARTer smRNA-Seq Kit for Illumina (TAKARA Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions. Small RNA sequencing was conducted commercially by Macrogen (Seoul, Republic of Korea) using a HiSeq 2500 System (Illumina Inc., San Diego, CA).

### Bioinformatic Analysis

Raw sequence reads were filtered using FastQC v0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and adapter sequences were trimmed from the raw sequence reads using Cutadapt 2.8 (https://cutadapt.readthedocs.org/en/stable/). The final processed reads were aligned against the chicken reference genome (GRCg6a), miRBase v22.1 (http://www.mirbase.org/) and RNAcentral release 14.0. The known and novel miRNAs were predicted using miRDeep2 (https://www.mdc-berlin.de/content/mirdeep2-documentation). Differentially expressed miRNAs (**DE**) were statistically analyzed based on fold changes and an exactTest using edge R (empirical analysis of digital gene expression data in R). Significant results were selected based on the criteria of a |fold-change| $\geq 2$ and exactTest raw $P$-value $< 0.05$. Target genes of the differentially expressed miRNAs were predicted using miRDB (http://www.mirdb.org/) and TargetScan (http://www.targetscan.org/vert_72/) databases. Target genes with a target score greater than 80 were selected using miRDB, as were genes with conserved sites determined using TargetScan. Gene ontology (**GO**) functional enrichment analysis of the target genes was performed using Gene Ontology Resource (http://geneontology.org/), and KEGG pathway enrichment analysis was conducted using DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/). miRNA target sites in genome sequences of A/duck/Vietnam/ QB1207/2012 (H5N1) (Accession numbers: PB2; KF182738.1, PB1; KF182739.1, PA; KF182740.1, HA; KF182741.1, NP; KF182742.1, NA; KF182743.1, M1 and M2; KF182744.1, NS1 and NS2; KF182745.1) and minimum free energy (**MFE**) were predicted using RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid).

**Table 1.** Sequences of the primers used for qRT-PCR analysis.

| MiRNA | Sequence (5'-3') |
|---|---|
| gga-miR-128-3p | TCACAGTGAACCGGTCTCTTT |
| gga-miR-142-5p | CCCATAAAGTAGAAAGCACTAC |
| gga-miR-193a-5p | TGGGTCTTTGCGGGCGAGATGA |
| gga-miR-16c-5p | TAGCAGCACGTAAATACTGGAG |
| U1A | CTGCATAATTTGTGGTAGTGG |

### Quantitative Real-Time-PCR Analysis of miRNA Expression

To validate the expression levels of sequenced genes, forward primers were designed for individual miRNAs and a universal reverse primer provided with a Mir-X miRNA FirstStrand Synthesis and TB Green Kit (Takara Bio, Japan). All known chicken miRNA sequences were obtained from miRBase (http://www.mirbase.org/). Oligonucleotide primers for these miRNAs were designed using full-length mature miRNA sequences. The primers were synthesized by Genotech (Daejeon, South Korea; Table 1). cDNA synthesis was performed using a Mir-X miRNA First-Strand Synthesis Kit (Takara Bio USA, Inc.) according to the manufacturer's protocol. A Mir-X miRNA qRT-PCR TB Green Kit (Takara Bio) was used to determine miRNA expression in the CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Relative levels of miRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with U1A, used as an internal control (Livak and Schmittgen, 2001). All qRT-PCR experiments were performed in triplicate.

### Statistical Analysis

Statistical analysis was performed using SPSS software (version 26.0; IBM, Chicago, IL). Data are expressed as the means ± SEM. Statistical comparisons were performed using Tukey's multiple comparison test, and the level of statistical significance was set at $P < 0.05$.

## RESULTS

### Analysis of Exosomal miRNA Expression

Exosomes purified from the blood serum of infected and noninfected chickens were characterized based on the exosomal markers CD9 and CD81 and size. CD9 and CD81 were detected in the exosomes of both control and infected birds using the corresponding antibodies (Figure 1A). The size range of exosomes obtained from both control and infected birds were within the range of 105 to 356 nm and average sizes were 141 nm (control) and 145 nm (infection) (Figure 1B).

Small RNA sequencing yielded 35,050,510 and 34,735,378 reads for the control and infected birds, respectively, which were aligned against the GRGg6a chicken reference genome using Bowtie (http://bowtie-bio.sourceforge.net/index.shtml) (Table 2). For control chickens, 81.58% (28,593,494) of the reads were mapped and for infected samples, 82.94% (28,808,111) of reads were mapped. To quantify mature miRNA abundance, the processed reads of both sample types were aligned using miRBase v22.1. Among the 1,235 known chicken miRNAs, we detected 138 and 143 in control and infected birds, respectively (Table 2).

The differential expression of miRNAs was determined based fold change and exactTest values using edgeR. Those showing a |fold change| ≥ 2 and exactTest raw $P$-value < 0.05 were selected as significantly differentially expressed miRNAs. Figure 2 shows a volcano plot of miRNA expression in infected and control chickens. Among the 107 detected miRNAs, we identified 10 miRNAs that were significantly differentially expressed,

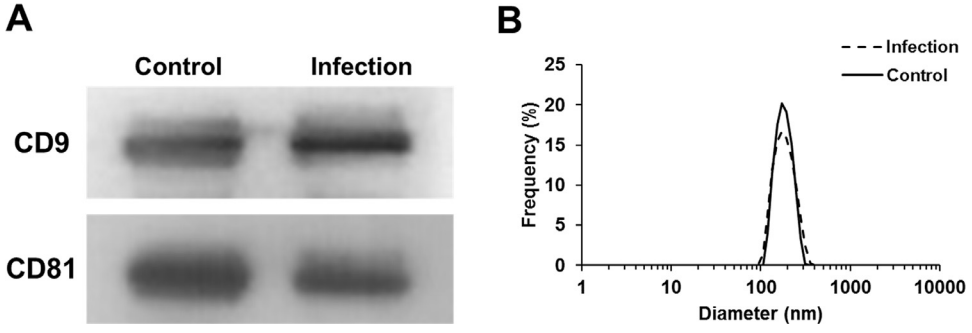


**Figure 1.** Characterization of purified exosomes. Exosomes derived from the blood serum of infected (**Infection**) and noninfected (**Control**) chickens were characterized in terms of (A) exosomal markers (CD9 and CD81) using western blotting and (B) particle size using a nanoparticle analyzer.

**Table 2.** Mapped reads to miRBase precursor.

| Sample | Total reads | Mapped reads | Known miRNA in Sample | Known miRNA in Species (miRBase v22.1) |
|---|---|---|---|---|
| Control | 35,050,510 | 28,593,494 (81.58%) | 138 | 1,235 |
| Infection | 34,735,378 | 28,808,111 (82.94%) | 143 | 1,235 |

Processed reads indicate reads which were trimmed and removed unwanted sources from them.

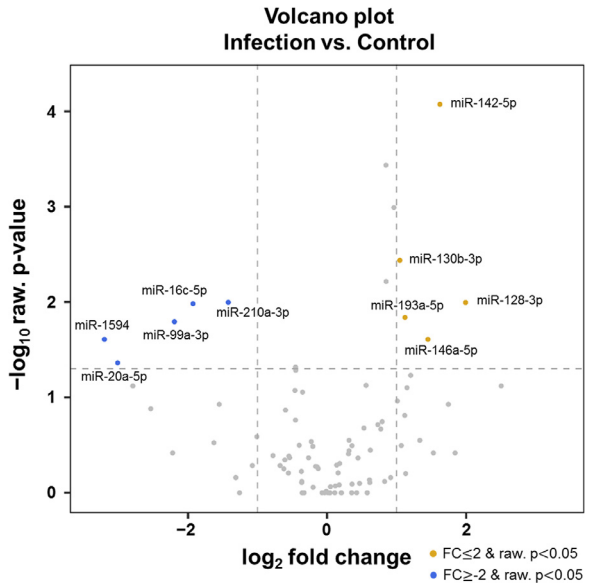


**Figure 2.** Volcano plot of the miRNAs expressed in exosomes. The plot shows the expression of exosomal miRNAs derived from noninfected (**Control**) and HPAIV H5N1-infected chickens (**Infection**). The X and Y axsis represent $\log_2$ fold changes (FCs) and $-\log_{10}$ *P*-values, respectively. The yellow and blue colors indicate FC $\geq 2$ and raw $P < 0.05$ and FC $\leq -2$ and raw $P < 0.05$, respectively.

5 of which (gga-miR-128-3p, gga-miR-142-5p, gga-miR-146a-5p, gga-miR-193a-5p, and gga-miR-130b-3o) were upregulated and 5 (gga-miR-210a-3p, gga-miR-16c-5p, gga-miR-99a-3p, gga-miR-20a-5p, and gga-miR-1594) were downregulated in infected birds compared with the controls. The fold changes of the miRNAs differentially expressed between infected and control chicken are shown in Figure 3. Among these DE miRNAs, Gga-miR-128-3p showed the highest upregulated fold of 3.97 and gga-miR-1594 showed the highest downregulated fold change of −9.20.

## Gene Ontology and KEGG Pathway Enrichment Analyses of Target Genes

The target genes of DE miRNAs were predicted using the miRDB and TargetScan databases. Totals of 1,873 and 3,042 genes were predicted as target genes using the miRDB and TargetScan databases, respectively

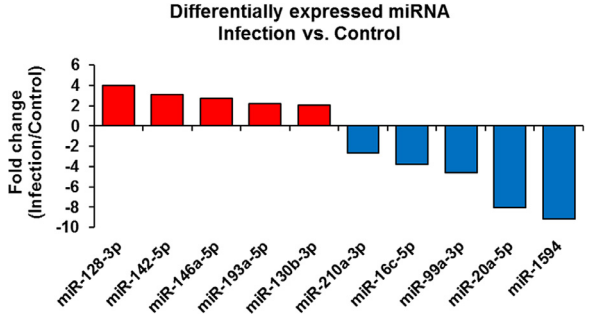


**Figure 3.** Fold changes of differentially expressed miRNAs. miRNAs differentially expressed between exosomes derived from infected (**Infection**) and noninfected (**Control**) chickens were selected based on the criteria of a |fold change| $\geq 2$ and exactTest raw *P*-value $< 0.05$.

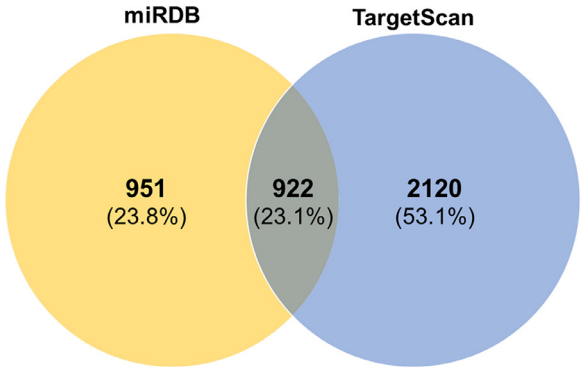


**Figure 4.** Venn diagram of the target genes of 10 differentially expressed miRNAs predicted using the miRDB and TargetScan databases. The numbers indicate predicted gene counts.

(Figure 4); of these, 922 that were commonly predicted as target genes using both databases were used for GO and KEGG pathway enrichment analyses. Figure 5 and Figure 6A shows the top 30 significantly enriched GO biological process, molecular function, and cellular component categories. In total, genes were assigned to 405, 71, and 42 GO terms in biological process, molecular function, and cellular component categories, respectively. In KEGG pathway enrichment analysis, target genes were found to be enriched in 20 KEGG pathways, with the MAPK signaling pathway being particularly enriched with the target genes of DE miRNAs (Figure 6B). Figure 7 presents a visualization of the interactions between DE miRNAs and their immune-related target genes, showing that a total of 268 immune-related genes (green) were predicted as target genes of DE miRNAs (red: upregulated DE miRNAs, blue: downregulated DE miRNAs). Notably, most immune-related genes (69) are regulated by gga-miR-16c-5p and several genes.

## Differentially Expressed miRNAs Target HPAIV RNA Segments

We speculated that DE miRNAs could play a role in inhibiting viral replication; to substantiate this supposition, we predicted the target sites of the 10 DE miRNAs in HPAIV H5N1 RNA segments using RNAhybrid. For each segment, target sites of miRNAs that showed lowest MFE values are indicated in Figure 8. Notably, we found that all HPAIV RNA segments were targeted by the 10 DE miRNAs. Table S1 presents information relating to sites in the virus genome targeted by miRNAs and the corresponding MFE values.

## qRT-PCR Analysis of Differentially Expressed miRNAs

To validate the small RNA sequencing results, we performed qRT-PCR analysis of the expression of selected miRNAs (gga-miR-128-3p, gga-miR-142-5p, gga-miR-193a-5p, and gga-miR-16c-5p; Figure 9). For infected birds, the expression levels of gga-miR-128-3p, gga-miR-

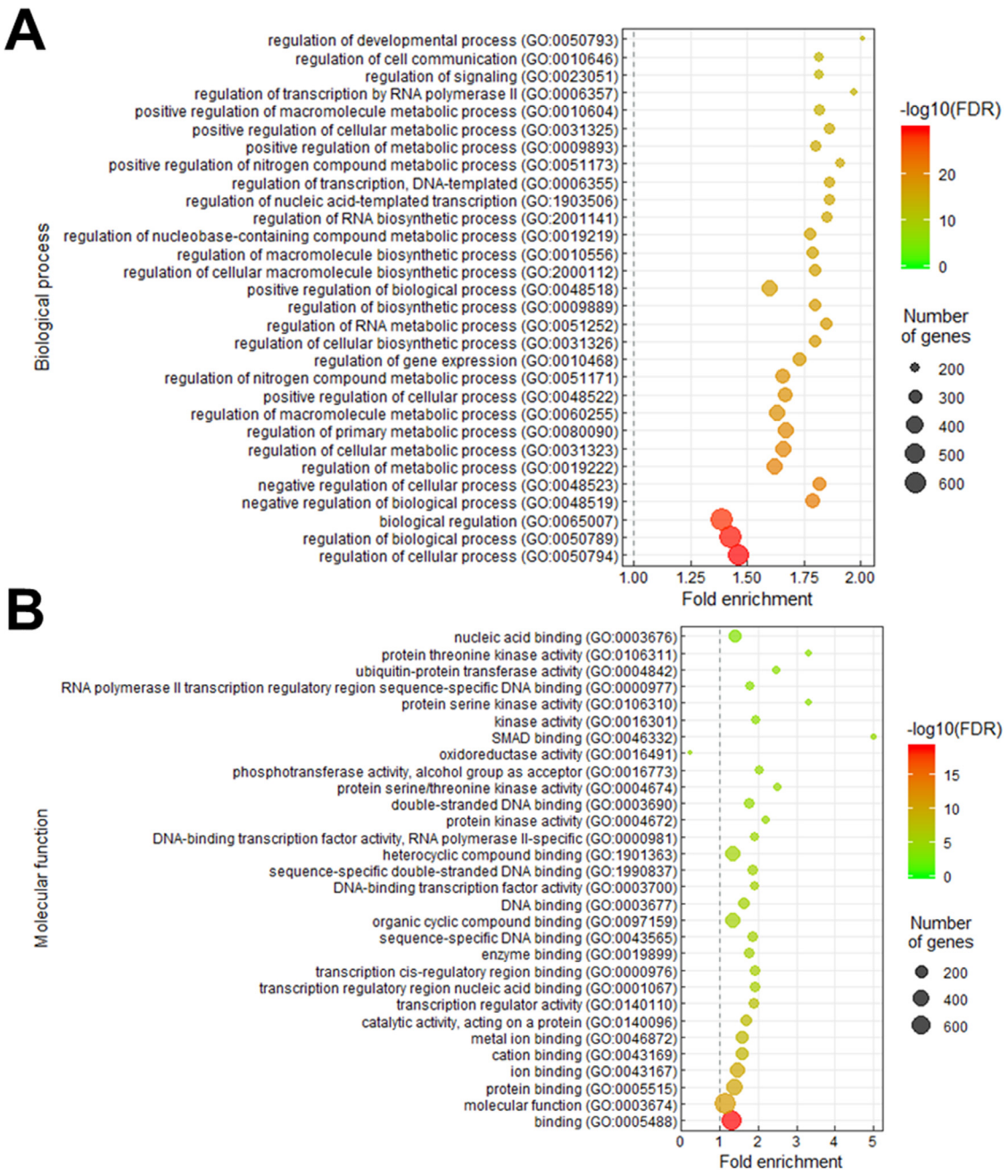


**Figure 5.** Gene Ontology enrichment analyses. Predicted target genes were used for Gene Ontology and KEGG pathway enrichment analysis and presented using the R program. The top 30 GO terms for the categories (A) biological process and (B) molecular function. The size of dots indicates the number of target genes associated with the GO term and the red and green dots indicate a low and high significance of enrichment [$-\log_{10}$(FDR)], respectively. The horizontal line represents the fold enrichment.

142-5p, gga-miR-193a-5p, and gga-miR-16c-5p were 2.74-, 3.08-, 1.49-, and 0.75-fold those in the uninfected birds, respectively. These expression patterns in infected and control samples were found to be consistent with the sequencing results.

## DISCUSSION

Exosomes promote intercellular communication via the delivery of exosomal contents such as proteins and miRNAs (Veerman et al., 2019). Notably in this regard, exosomal miRNAs can contribute to the regulation of biological processes in recipient cells by inhibiting target gene expression (Zhang et al., 2015). Consequently, in the present study, we sought to characterize exosomal chicken miRNA profiles with respect to the response to HPAIV H5N1 infection based on small RNA sequencing. We accordingly identified 10 miRNAs that were significantly differentially expressed between infected and noninfected White Leghorn chickens, 5 of which were up- and downregulated, respectively, in exosomes derived from H5N1-infected chickens. Moreover, we established that most of the target genes of these 10 DE miRNAs are associated with MAPK signaling pathway (Figure 6B). Moreover, we found that these DE miRNAs target HPAIV H5N1 RNA segments (Figure 8).

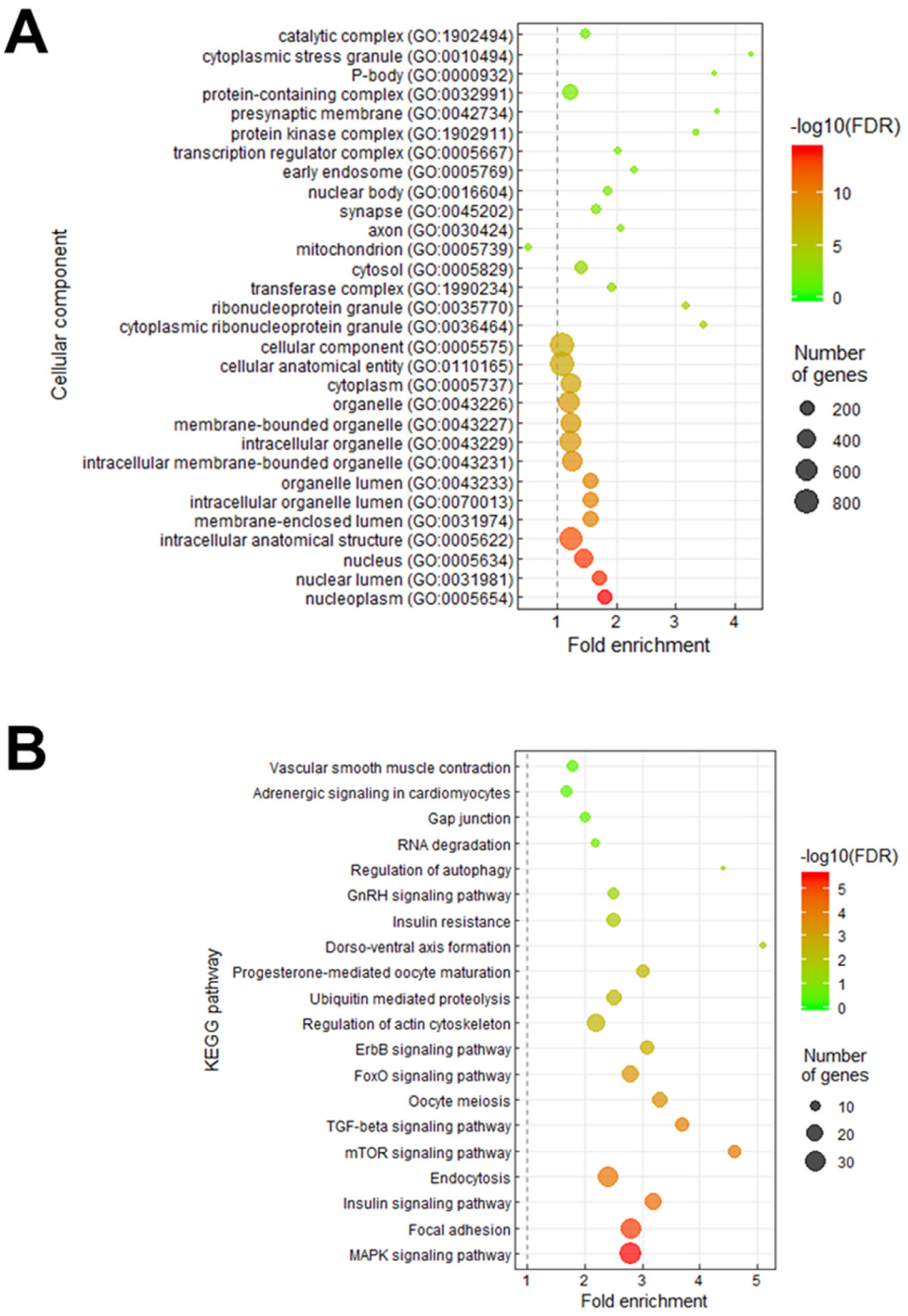


**Figure 6.** Gene Ontology and KEGG pathway enrichment analyses. Predicted target genes were used for Gene Ontology and KEGG pathway enrichment analysis and presented using the R program. The top 30 GO terms (A) cellular component and (B) KEGG pathway enrichment analysis. The size of dots indicates the number of target genes associated with the GO term and the red and green dots indicate a low and high significance of enrichment [$-\log_{10}$(FDR)], respectively. The horizontal line represents the fold enrichment.

Among the 10 DE miRNAs, several have been studied previously with respect to viral infection and the immune system. For example, overexpression of gga-miR-142-5p has been shown to promote infectious replication of bursal disease virus (**IBDV**) by targeting the melanoma differentiation-associated 5 (***MDA5***) gene, an RNA-recognizing pattern recognition receptor (Ouyang et al., 2018), whereas upregulation of miR-146a-5p induced by hepatitis A virus (**HAV**) has been found to repress the expression of IFN-$\beta$ and promote viral replication by targeting TNF receptor-associated factor 6 (***TRAF6***; Mo et al., 2021). Similarly, IBDV induces the upregulation of gga-miR-130b-3p, which in turn inhibits IBDV by targeting the IBDV genome and suppressor of cytokine signaling 5 (***SOCS5***) (Fu et al., 2018). Moreover, the upregulated expression of miR-130b-3p in response to *Mycoplasma gallisepticum* has been found to activate the PI3K/AKT/NF-$\kappa$B pathway, an essential pathway involved in the regulation of immune responses (Yuan et al., 2018), and Gga-miR-16c-5p is downregulated in Reticuloendotheliosis virus-infected chickens (Yu et al., 2017), as well as in duck

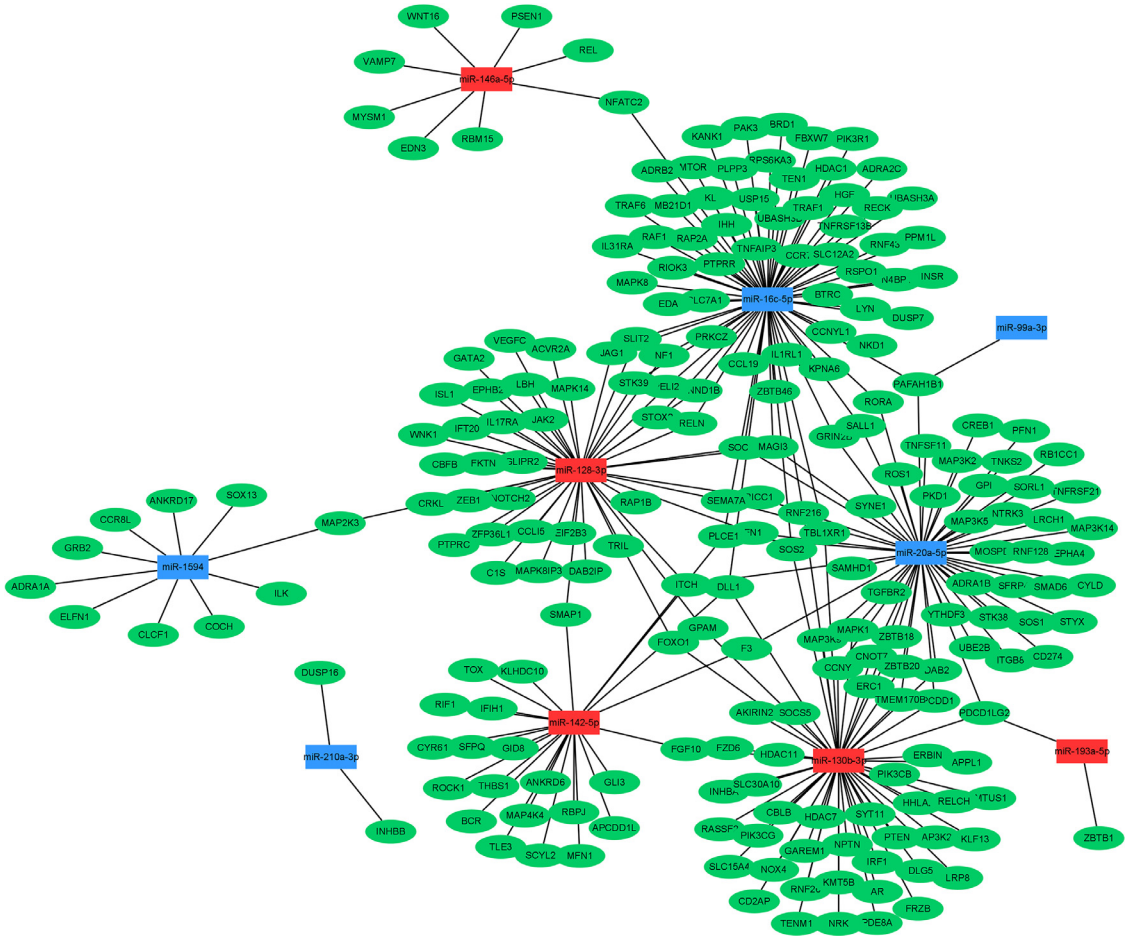


**Figure 7.** Network of differentially expressed miRNAs and immune-related target genes. The interaction of differentially expressed miRNAs and immune-related target genes was visualized using Cytoscape. The red and blue square nodes indicate the up- and downregulated differentially expressed miRNA in infection, respectively. The green circular nodes represent the immune-related target genes.

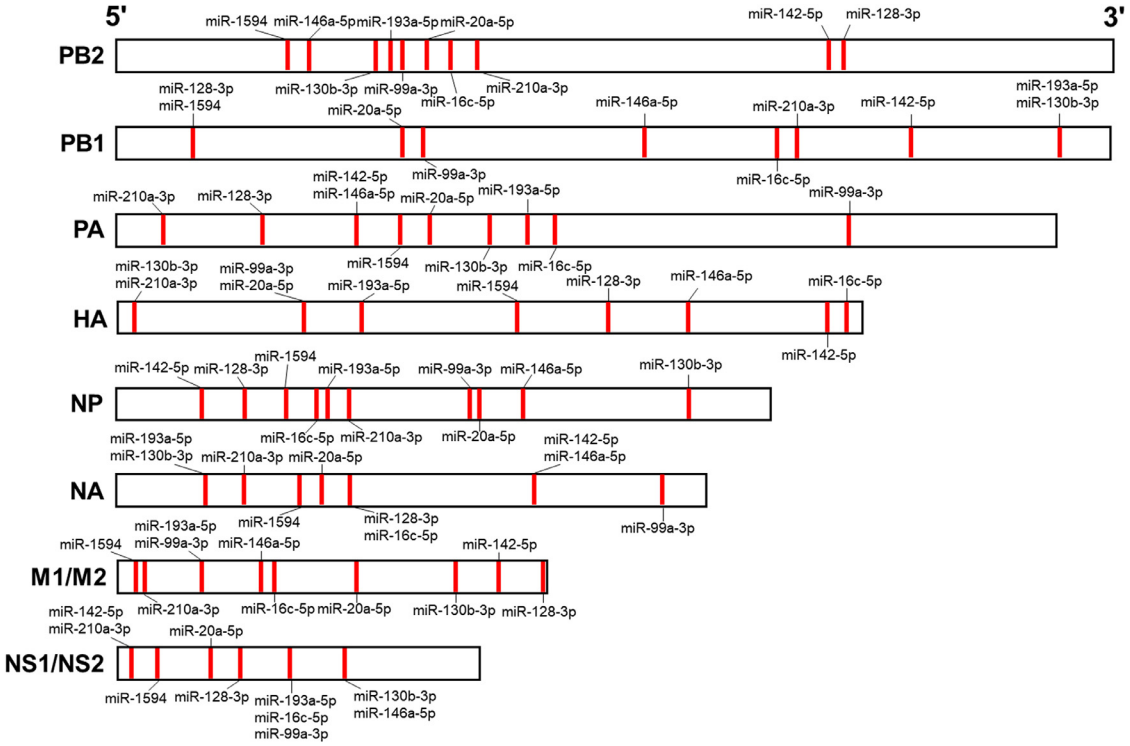


**Figure 8.** The differentially expressed miRNAs target HPAIV H5N1 viral RNA segments. MicroRNA binding sites in HPAIV H5N1 viral RNA segments were predicted using RNAhybrid. The red color represents sites targeted by the indicated miRNAs.

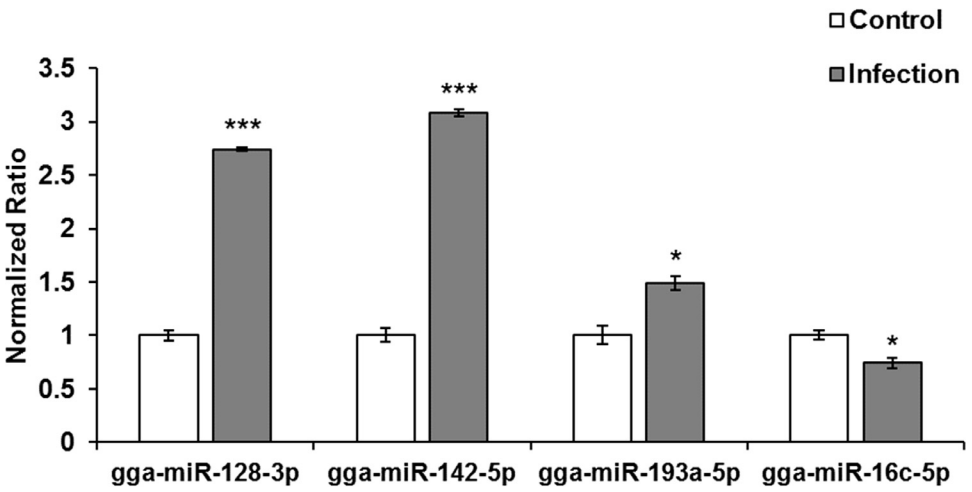


**Figure 9.** Validation of sequencing results by qRT-PCR analysis. The expression of exosomal miRNAs were analyzed using qRT-PCR. The expression of miRNAs was normalized by expression of the internal control. Data are expressed as the means ± SEM of three independent experiments: $^{*}P < 0.05$ and $^{***}P < 0.001$.

enteritis virus-infected duck embryo fibroblast cells (Wu et al., 2019). On the basis of these findings, we speculate that the DE miRNAs identified in the present study might play pivotal roles in regulating the replication of the highly pathogenic avian influenza virus and the corresponding host immune response.

Our KEGG pathway enrichment analysis revealed that the target genes of most DE miRNAs identified in this study are associated with the MAPK signaling pathway (Figure 6B). Upon HPAIV infection, viral genomes are recognized by Toll-like receptor (**TLR**) 3 or TLR7, thereby activating the NF-$\kappa$B and MAPK signaling pathways, which in turn promote the production of proinflammatory cytokines via a cascade process (Yu et al., 2020). Accordingly, we suggest that regulation of the MAPK signaling pathway by exosomal miRNAs constitutes one of the most important mechanisms underlying the defense against HPAIV infection.

Several studies have reported that miRNAs inhibit the replication of HPAIV by targeting its genomes. For example, miR-324-5p and miR-3145 have been demonstrated to inhibit the replication of H5N1 by targeting the PB1 segment of this virus (Khongnomnan et al., 2015; Kumar et al., 2018). Similarly, miR-584-5p and miR-1249 have been shown to repress the replication of H5N1 by targeting the PB2 segment (Wang et al., 2017). In the present study, we predicted that the 10 characterized DE miRNAs target eight RNA segments of the H5N1 genome. In this regard, we suspect that the intercellular delivery of exosomal miRNAs to virus-infected cells could be mediated via biological fluids, wherein they inhibit viral replication by targeting the virus genome. However, further studies will be necessary to determine whether this putative mechanism actually inhibits viral propagation.

In summary, we examined the expression of exosomal miRNAs in noninfected and HPAIV H5N1-infected White Leghorn chickens. We accordingly identified 10 miRNAs that were differentially expressed in exosomes derived from infected birds compared with those from noninfected birds. Most of the genes targeted by these differentially expressed miRNAs were found to be involved in regulation of the MAPK signaling pathway, with several being identified as immune-related. Furthermore, we predicted that the 10 differentially expressed miRNAs would bind to RNA segments in the HPAIV H5N1 genome, and thereby inhibit its replication. Collectively, the findings of this study will contribute to gaining an understanding of the regulatory mechanisms of exosomes in the host response to HPAIV H5N1 infection, and also provide information that can be utilized in the development novel vaccines for the prevention of HPAIV H5N1 infection.

## ACKNOWLEDGMENTS

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## DISCLOSURES

The authors declare that they have no competing interests.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102123.

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