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Research Article

miR-204 suppresses porcine reproductive and respiratory syndrome virus (PRRSV) replication via inhibiting LC3B-mediated autophagy

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) caused by PRRS virus (PRRSV) has been regarded as a persistent challenge for the swine farms worldwide. microRNAs (miRNAs) play key roles in regulating almost every important biological process, including virus-host interaction. In this study, we found that miR-204 was highly expressed in cells that were not permissive to PRRSV infection compared with cells susceptible to PRRSV infection. Subsequently, we demonstrated that overexpression of miR-204 significantly inhibited PRRSV replication in porcine alveolar macrophages (PAMs). Through bioinformatic analysis, we found that there existed a potential binding site of miR-204 on the 3′UTR of microtubule associated protein 1 light chain 3B (MAP1LC3B, LC3B), a hallmark of autophagy. Applying experiments including luciferase reporter assay and UV cross-linking and immunoprecipitation (CLIP) assay, we demonstrated that miR-204 directly targeted LC3B, thereby down-regulating autophagy. Meanwhile, we investigated the interplay between autophagy and PRRSV replication in PAMs, confirming that PRRSV infection induces autophagy, which in turn facilitates viral replication. Overall, we verify that miR-204 suppresses PRRSV replication via inhibiting LC3B-mediated autophagy in PAMs. These findings will provide a novel potential approach for us to develop antiviral therapeutic agents and controlling measures for future PRRSV outbreaks.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS), also known as “blue ear” disease, is an infectious disease that leads to reproductive failures in pregnant sows and severe respiratory syndromes in pigs of all ages (You et al., 2022). It has threatened the global pig industry and caused tremendous economic losses since its outbreak in the late 1980s (Dhakal and Renukaradhya, 2019; Pileri and Mateu, 2016). PRRS is caused by porcine reproductive and respiratory syndrome virus (PRRSV), a single-stranded positive-sense enveloped RNA virus, which belongs to the family *Arteriviridae* in the order *Nidovirales* (Meulenberg, 2000). The PRRSV genome is approximately 15.4 kb in length and contains at least 11 open reading frames (ORFs), encoding 8 structural proteins and at least 16 non-structure proteins. According to sequence analysis, PRRSV is mainly divided into PRRSV-1 (species *Betaarterivirus suid 1*) and PRRSV-2 (species *Betaarterivirus suid 2*) (Wang et al., 2021). The nucleotide sequence similarity between PRRSV-1 and PRRSV-2 is about 50%–70% (Kappes and Faaberg, 2015). In 2006, an atypical PRRS caused by highly pathogenic PRRSV (HP-PRRSV) suddenly broke out in China and quickly spread to most areas of Asia, which has made the pig industry face great challenges (Chen et al., 2021). Due to the continuous recombination and mutation of PRRSV, the main prevention and control measures against PRRS, such as vaccines, cannot provide a satisfactory protective role (Zhang et al., 2022). Hence, it is an urgent need to seek new targets that can regulate PRRSV infection and help us find better design of therapeutic approaches and preventive measures to minimize PRRS impacts on the swine industry.

microRNAs (miRNAs) are a conserved class of endogenous non-coding single-stranded RNAs, which consist of approximately 22 nucleotides and regulate gene expression post-transcriptionally. In mammals, miRNAs play key roles in several biological processes, including cell differentiation, metabolism and apoptosis (Rhman and Pmo, 2022). Furthermore, numerous studies have demonstrated the widespread importance of miRNAs in host-virus interaction. miRNAs participate in modulating infection and replication of multiple viruses. For instance, miR-133a suppresses dengue virus (DENV) replication, likely through interference with polypyrimidine tract binding protein (PTB) expression (Castillo et al., 2016). miR-323, miR-491 and miR-654 resist the

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replication of H1N1 influenza A virus (IAV) via binding to the viral RNA polymerase PB1 subunit gene (Keshavarz et al., 2018).

Autophagy is a conserved cellular recycling mechanism in eukaryocytes, which degrades long-lived cytoplasmic proteins and damaged organelles to maintain intracellular homeostasis (Klionsky, 2008). Many intracellular and extracellular stresses, such as nutrient deprivation, growth factor depletion, infection, and hypoxia, can induce the autophagic response (Dikic and Elazar, 2018). Besides the physiological functions of autophagy, accumulating evidence has indicated that autophagy plays a dual role in the infection processes of diverse pathogens. As an irreplaceable component of the human immune defense system, autophagy is essential in the face of viral invasion, whereby cells can eliminate viral proteins, nucleic acids and viral particles through autophagy to maintain the health of the body (Levine and Kroemer, 2019; Yang and Klionsky, 2020). However, some viruses have evolved mechanisms to evade host cellular autophagy and even hijack autophagy to facilitate their own replication, such as PRRSV, grass carp reovirus (GCRV), and pseudorabies virus (Xu et al., 2018; Zhou et al., 2016; Zhu et al., 2022).

In this study, we compared the miRNA profiles in PRRSV target cells (porcine alveolar macrophages, PAMs) and non-permissive cells (porcine peritoneal macrophages, PPMs) to identify differentially expressed miRNAs (DEmiRNAs), and found that miR-204 was highly expressed in PPMs and lowly expressed in PAMs. We then showed that overexpression of miR-204 inhibited PRRSV replication in a dose-dependent manner. Subsequently, we demonstrated that miR-204 directly targeted microtubule associated protein 1 light chain 3B (MAP1LC3B, LC3B), which in turn suppressed autophagy induced by PRRSV infection and rapamycin (an autophagy agonist). Therefore, we conclude that miR-204 is a novel repressor of PRRSV replication and may provide us with a new therapeutic target for controlling PRRS in the future.

2. Materials and methods

2.1. Cells and viruses

PAMs were obtained from lung lavage of 6−8-week-old specific-pathogen-free (SPF) pigs and cultured in RPMI 1640 medium (Gibco, USA) with additional 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). Porcine peritoneal macrophages (PPMs) were isolated from the peritoneal lavage of SPF pigs and maintained with the same culture condition as PAMs. 3D4/21 cells (ATCC number: CRL-2843 cells), a porcine alveolar macrophage cell line, were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS and 1% penicillin-streptomycin. All of the cells were cultured at 37 °C with 5% CO_2.

The HP-PRRSV isolate HV (GenBank Accession No. JX317648) and classical PRRSV isolate VR2332 (GenBank Accession No. U87392) were propagated on PAMs and stored at −80 °C until use.

2.2. Reagents and antibodies

Rabbit monoclonal antibody against LC3B (#43566) was purchased from Cell Signaling Technology (USA). Mouse monoclonal β-actin antibody was purchased from Sigma-Aldrich (USA). Ago2 protein monoclonal antibody (#H00027161-M01) was purchased from Abnova (China). PRRSV nucleocapsid (N) antibody was prepared in our laboratory. Goat anti-mouse secondary antibody (#SE131) and goat anti-rabbit secondary antibody (#SE134) were purchased from Solarbio (China). The activator and inhibitor of autophagy, rapamycin (#HY-10219) and 3-methyladenine (3-MA) (#HY-19312), were purchased from MedChemExpress (USA). HiperFect transfection reagent was purchased from QIAGEN (Germany).

The miRNA mimics, negative control (NC) mimics, and siRNAs were synthesized by Genepharma (China) and listed in Supplementary Table S1.

2.3. Small RNA deep sequencing

We obtained PAMs and PPMs from lung and peritoneal lavage of SPF pigs, and cultured cells at 37 °C with 5% CO_2 for 24 h. Then, cells were collected and the TRIzol reagent (Invitrogen, USA) was used to isolate total cellular RNA from PAMs and PPMs. Then, the differential expression of miRNAs was analyzed by deep sequencing, which was performed using an Illumina Genome Analyzer at LC Sciences (Houston, USA). The expression of miRNAs was analyzed by calculating fold-change. miRNAs were labeled as differentially expressed, when the fold change was ≥ 2 and P value was ≤ 0.01.

2.4. Transfection and quantitative real-time PCR

According to the instructions, all the miRNA mimics, NC mimics or siRNAs were transfected into PAMs using HiPerFect transfection reagents (QIAGEN). Meanwhile, NC mimics were used as the matched controls.

Total RNAs were extracted from cells with TRIzon Reagent (CWBIO, China). HiFiScript cDNA Synthesis Kit (CWBIO, China) was used to reverse transcribe 1 μg of RNAs into cDNAs according to the manufacturer's instructions. Quantitative real-time PCR analysis was performed in a ViiA 7 real-time PCR System (Applied Biosystems) with SYBR Green real-time PCR Master Mix (Mei5 Biotechnology, China). Gene-specific primers were listed in Supplementary Table S2.

To quantify miRNAs, we used Hairpin-it™ Real-time PCR kit (Genepharma, China). The $2^{-\Delta\Delta CT}$ Ct method was used to calculate the relative expression of the target gene. The expression of mRNAs was normalized to GAPDH, while miRNAs were normalized to U6.

2.5. Indirect immunofluorescence assay (IFA)

PAMs were transfected with NC or miRNA mimics for 12 h followed by infection with HP-PRRSV isolate HV at an MOI of 0.01. At 36 h later, cells were fixed in cold methanol-acetone (1:1) for 10 min at 4 °C, washed three times with phosphate buffered saline (PBS), and then blocked with 5% goat serum in PBS for 30 min at room temperature. Then, cells were incubated with anti-PRRSV N protein monoclonal antibody at 37 °C for 1 h, followed by washing three times with PBS. Next, cells were incubated with FITC-conjugated goat anti-mouse IgG antibodies for 1 h at 37 °C. After washing three times with PBS, cells were examined using fluorescence microscopy (IX71, Olympus Corporation, Japan).

2.6. Western blot

Cells were lysed with RIPA lysis buffer (CWBIO, China) supplemented with a protease inhibitor mixture (Mei5 Biotechnology, China). Proteins were separated on SDS-PAGE gels and then transferred onto polyvinylidene fluoride membranes (Millipore, USA). Membranes were then blocked with 5% skim milk in PBST (PBS with 0.1% Tween-20) for 1 h at room temperature, and then incubated with anti-PRRSV N (1:1000), anti-LC3B (1:1000), or β-actin (1:5000) for 1 h at room temperature. After washing three times, the membranes were incubated with appropriate secondary antibody (1:5000) for 1 h at room temperature. After washing, the antibodies were visualized using chemiluminescence (ECL; CWBIO, China) reagent. β-actin was used as a loading control.

2.7. Plasmid construction and luciferase reporter assays

To verify whether LC3B or ATG7 is targeted by miR-204, the predicted target site in the porcine *LC3B* 3′UTR (282 bp) or *ATG7* 3′UTR (299 bp) was amplified and inserted into the C-terminus of the firefly luciferase gene in the pGL3-control vector (Promega, USA) to create wild type 3′UTR vector (LC3B-3′UTR-wt or ATG7-3′UTR). A mutant vector (LC3B-3′UTR-mut) was constructed by mutating four seed nucleotides using a site-directed mutagenesis kit (NEB, USA) according to the

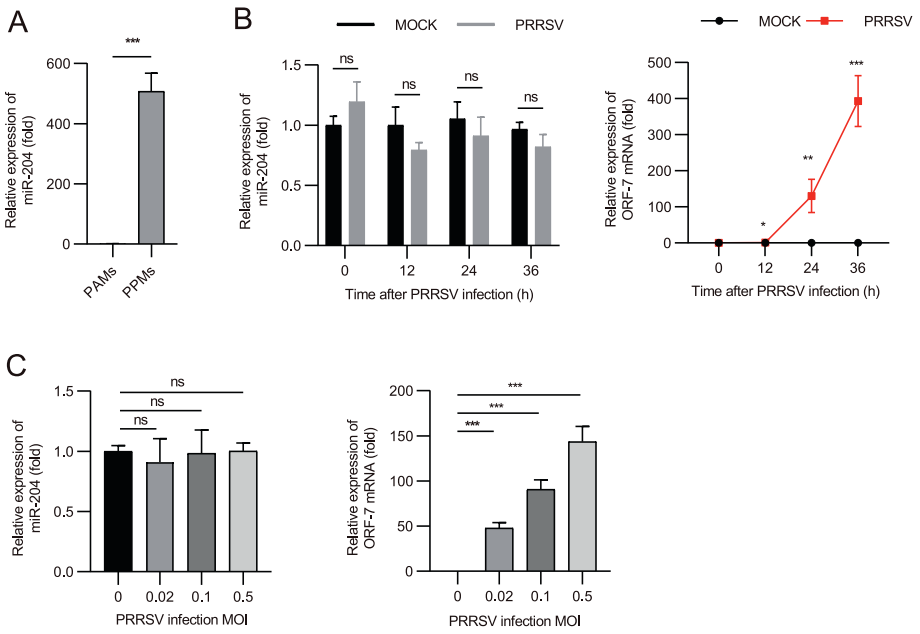

Fig. 1. The expression of miR-204 remains unchanged during PRRSV infection. **A** PAMs and PPMs were isolated from SPF pigs and the expression of miR-204 was detected by RT-qPCR. **B** PAMs were infected with HP-PRRSV isolate HV (MOI = 1) for the indicated times, and the expression of miR-204 and PRRSV ORF7 was analyzed by RT-qPCR, respectively. **C** PAMs were either mock or infected with HV at an MOI of 0.02, 0.1 or 0.5 for 24 h, and then cells were harvested for the analysis of miR-204 and PRRSV ORF7 expressions by RT-qPCR. Results are expressed as means ± standard deviation from three independent experiments. *P* values were analyzed using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

manufacturer's instructions. Gene-specific primers were listed in Supplementary Table S3.

3D4/21 cells were co-transfected with pGL3-control vector, pRL-TK, or miRNAs for 36 h. Cell extracts were prepared and analyzed for firefly and Renilla luciferase activities using a dual-luciferase reporter assay kit (Promega, USA) according to the manufacturer's protocol.

2.8. UV cross-linking and immunoprecipitation (CLIP)

PAMs were plated on 6-well culture plates and then transfected with NC or miR-204 mimics using HiPerFect reagents for 30 h. Next, cells were cross-linked with UV and lysed with lysis buffer supplemented with a protease inhibitor mixture. The anti-Ago2 monoclonal antibody or isotype control IgG was mixed with protein A-agarose (Sigma, USA) for 2 h, and then incubated with the supernatants of cell lysate at 4 °C overnight. The RNA from the immunoprecipitation product was isolated by using TRIzol and quantified by RT-qPCR.

2.9. Viral titration assay

The supernatants from cell cultures were collected at the indicated time points after virus inoculation, and fifty percent tissue culture infective dose ($TCID_{50}$) assay was performed to assess viral titration as described previously (Zhang et al., 2005) with minor modifications. Firstly, PAMs were plated on 96-well culture plates for 12 h. Then cells were infected with ten-fold serial dilution of the indicated viral supernatants, and 100 mL of the dilutions was added per well in replicates of eight. Finally, 3 or 4 days later, the viral titers were determined using the Reed-Muench method and expressed as $TCID_{50}$.

2.10. miRNA target prediction

The online databases, TargetScan (https://www.targetscan.org/vert_80/) and miRPathDB (https://mpd.bioinf.uni-sb.de/), were used to predict the targets of miR-204. The gene sequences of LC3B 3′UTRs of different species were obtained from NCBI (https://www.ncbi.nlm.nih.gov/). SnapGene software (version 3.2.1, www.snapgene.com) was used to compare sequences.

2.11. Statistical analysis

All the experiments were conducted with at least three independent replicates. The results were analyzed using GraphPad Prism software (version 8.0.2, www.graphpad.com). Differences between the data were analyzed using Student *t*-test. Significance was denoted as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and ns, not significant.

3. Results

3.1. miR-204 suppresses PRRSV replication

PRRSV is highly restricted to mononuclear-macrophage lineage. It can replicate efficiently in PAMs but not in PPMs (Duan et al., 1997). Our previous studies have shown that DEmiRNAs in PAMs and PPMs may play roles in regulating PRRSV infection and replication. miR-142-3p, which is expressed higher in PPMs and lower in PAMs, inhibits PRRSV infection by directly targeting Rac1 in PAMs (Yao et al., 2022). Another DEmiRNA, miR-150, suppresses PRRSV replication by targeting viral genome and SOCS1 (Li et al., 2022). Here, we performed high-throughput sequencing of small RNAs and identified miR-204 as a candidate, which is expressed higher in PPMs than in PAMs. For further verification, we examined the expression of miR-204 by RT-qPCR and confirmed that miR-204 expression was at higher levels in PPMs, compared to PAMs (Fig. 1A). Since viral infection may influence various miRNA expression in host cells, we performed a PRRSV-infected time-course experiment and a virus dose-dependent experiment in PAMs and analyzed the expression of miR-204 by RT-qPCR during PRRSV infection. Our results showed that PRRSV had no effect on the expression of miR-204 in PAMs (Fig. 1B and C), suggesting that PRRSV infection does not modulate miR-204 expression.

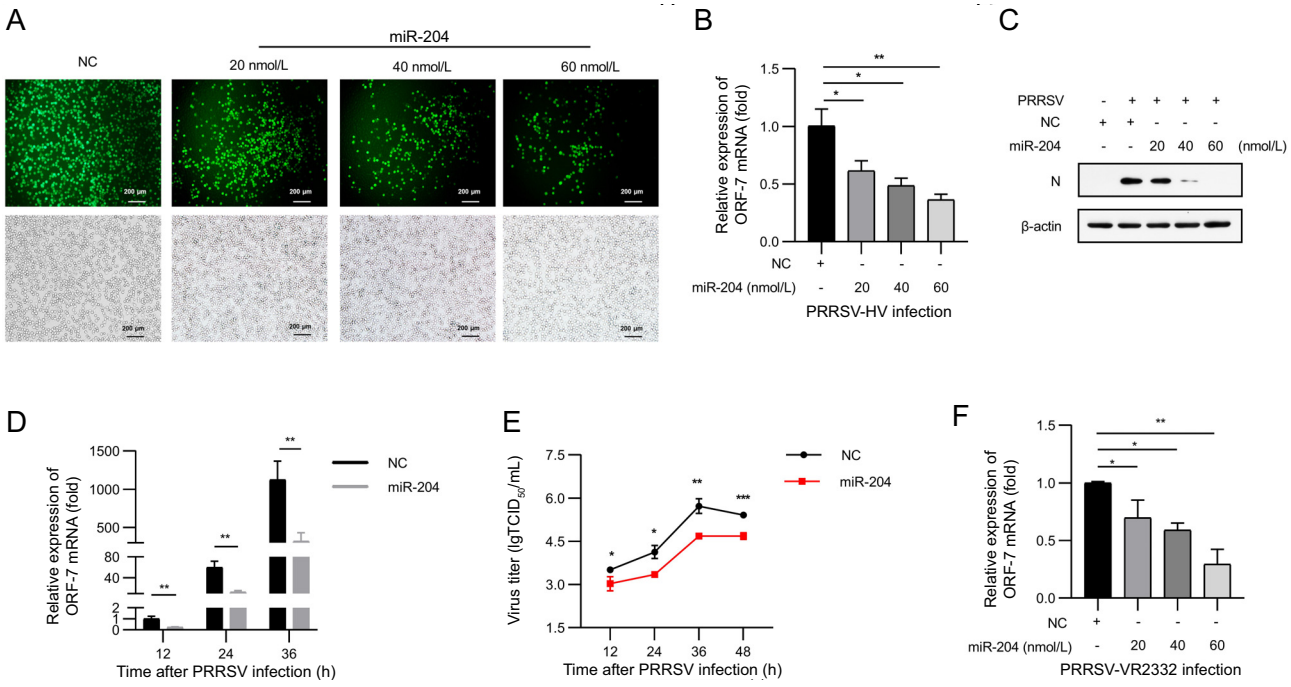

Fig. 2. miR-204 impairs PRRSV replication. **A–C** PAMs were transfected with negative control (NC) or miR-204 mimics at the indicated concentrations for 12 h, followed by infection with HP-PRRSV isolate HV (MOI = 0.01) for 36 h. Then, cells were fixed for immunofluorescent staining of PRRSV N protein (scale bar = 200 μm) (**A**), harvested for PRRSV ORF7 expression by RT-qPCR (**B**), or collected for PRRSV N protein detection by Western blot (**C**). **D, E** PAMs were transfected with NC or miR-204 mimics at a final concentration of 60 nmol/L for 12 h, followed by infection with HP-PRRSV isolate HV (MOI = 0.01) for the indicated times. The expression of PRRSV ORF7 was analyzed by RT-qPCR (**D**), and the virus titers were determined by $TCID_{50}$ (**E**). **F** PAMs were transfected with NC or miR-204 mimics at the indicated concentrations for 12 h, followed by infection with classical PRRSV isolate VR2332 (MOI = 0.01) for 36 h. The expression of PRRSV ORF7 was analyzed by RT-qPCR. Results are expressed as means ± standard deviation from three independent experiments. *P* values were analyzed using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

To investigate the role of miR-204 in the regulation of PRRSV replication, we transiently transfected miR-204 mimics or NC mimics into PAMs for 12 h and then infected the cells with HP-PRRSV isolate HV (MOI = 0.01). After 36 h, cells were harvested for indirect immunofluorescence assay (IFA). The results showed that miR-204 significantly inhibited PRRSV replication in a dose-dependent manner (Fig. 2A). Meanwhile, we performed RT-qPCR to detect the expression of PRRSV ORF7. Our results indicated that overexpression of miR-204 resulted in a remarkable decrease of PRRSV ORF7 mRNA by around 40%, 60%, and 70% when miR-204 mimics were at a concentration of 20, 40, and 60 nmol/L, respectively (Fig. 2B). Western blot also verified the inhibitory effect of miR-204 on PRRSV replication (Fig. 2C). To further clarify the function of miR-204, we tested the effect of miR-204 on PRRSV replication at different time points after PRRSV infection. Our results revealed that miR-204 suppressed PRRSV replication at the indicated time points and the inhibitory effect reached a peak at 36 h post-infection (Fig. 2D). Subsequently, $TCID_{50}$ assay was applied to monitor the dynamics of PRRSV replication in PAMs overexpressed with miR-204. The data showed that viral growth was repressed about 10 folds compared to NC at 36 h post-infection (Fig. 2E). To examine whether the inhibitory effect of miR-204 on PRRSV replication is strain-dependent, we infected miR-204-transfected PAMs with classical PRRSV isolate VR2332. The data verified that miR-204 also impaired PRRSV VR2332 replication (Fig. 2F). Taken together, these results indicate that miR-204 suppresses PRRSV replication.

3.2. *miR-204 directly targets LC3B*

To explore the mechanism of miR-204 in the regulation of PRRSV replication, we used miRNA target-prediction algorithms, TargetScan and miRPathDB, to predict its specific targets. The analysis showed that miR-204 had multiple potential targets involved in different life activities. Since the existing studies have demonstrated that PRRSV can use the autophagic machinery to promote its replication, we then focused on the targets associated with autophagy. We found that autophagy related 7 (ATG7), a key regulator of autophagy, was a possible target of miR-204. Analysis revealed that there was a putative target site for miR-204 in ATG7 3′UTR (Supplementary Fig. S1A). However, we demonstrated that miR-204 did not target ATG7 (Supplementary Fig. S1B).

Meanwhile, we found that LC3B was also a predicted target of miR-204. LC3B is required for autophagosome formation and maturation, and is currently the most widely used molecular marker to monitor autophagy (Klionsky et al., 2021). Bioinformatics analysis uncovered that miR-204 could bind to 3′UTR of LC3B through a seed match interaction and the putative target site was conserved across different species (Fig. 3A). To verify the relationship between LC3B and miR-204, we amplified the predicted target site in the LC3B 3′UTR and inserted it into a firefly luciferase reporter vector (LC3B-3′UTR-wt). In addition, a mutant vector (LC3B-3′UTR-mut) was constructed to eliminate the possible recognition by replacing four seed nucleotides (AAAGGGA to UAUGCGU) (Fig. 3A). Each of the vectors was co-transfected with miR-204 mimics into 3D4/21 cells (a cell line derived from porcine alveolar macrophage), and the luciferase activity was analyzed. The results indicated that miR-204 reduced the luciferase activity of LC3B-3′UTR-wt, but did not influence the activity of LC3B-3′UTR-mut (Fig. 3B). In addition, we proved that miR-204 suppressed the luciferase activity of LC3B-3′UTR-wt in a dose-dependent manner (Fig. 3C). The inhibitory effect of miR-204 was also verified by Western blot. As shown in Fig. 3D, ectopic expression of miR-204 decreased the protein level of LC3B. To further prove LC3B as a target of miR-204, we mutated the seed region of miR-204 and performed the same experiments. As predicted, miR-204 mutants had no effects on the luciferase activity of LC3B-3′UTR-wt or expression of LC3B protein (Fig. 3E and F). These data suggest that miR-204 targets LC3B to downregulate its expression.

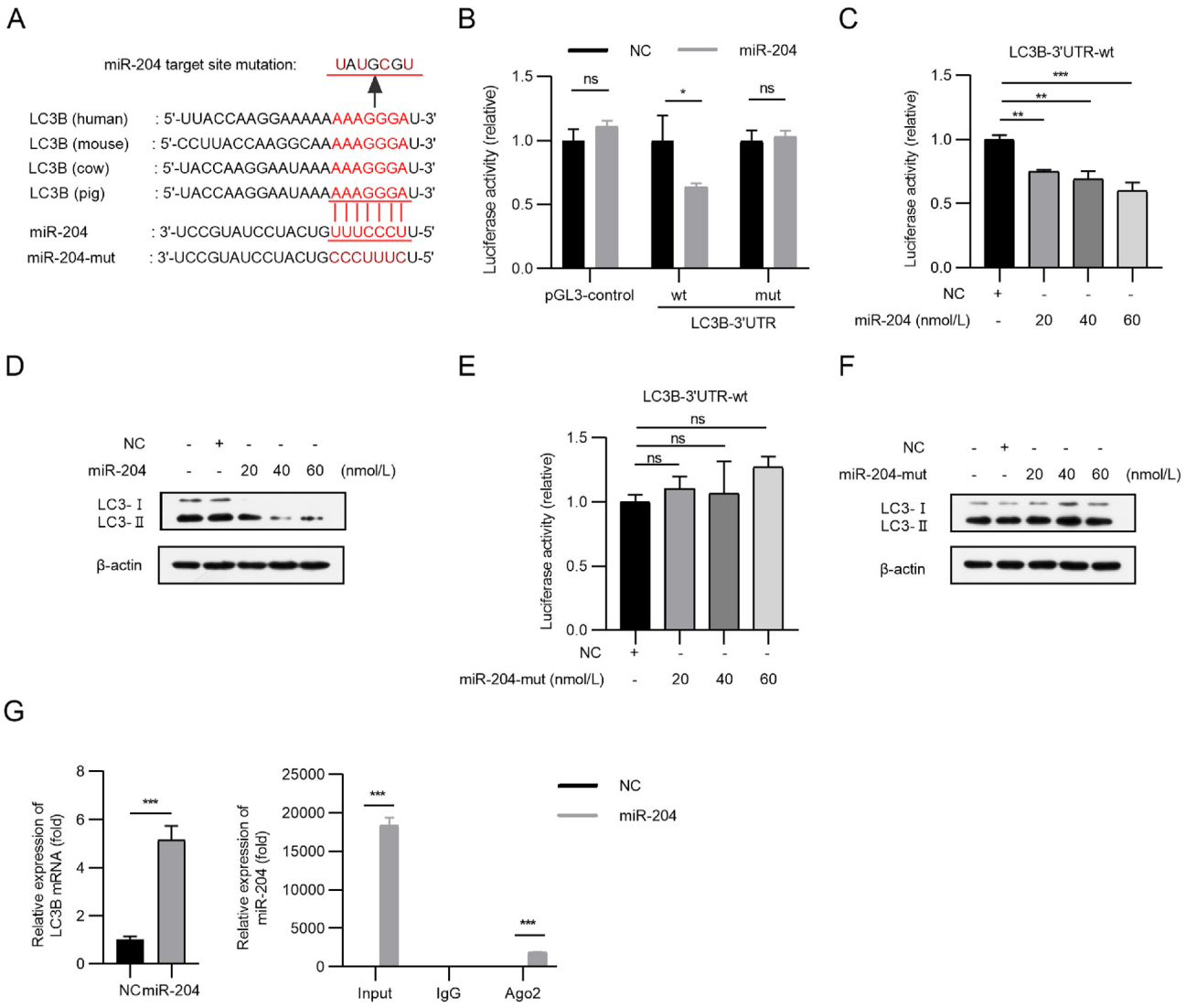

Fig. 3. miR-204 directly targets LC3B. **A** Schematic presentation of base pairing between the 3′UTR of LC3B and miR-204 sequence. TargetScan and miRPathDB were used to predict the targets of miR-204. The gene sequences of LC3B 3′UTRs of different species were obtained from NCBI and analyzed by SnapGene software. The underlined red bases are their paired bases and their corresponding mutant bases. **B** 3D4/21 cells were co-transfected with LC3B-3′UTR-wt or LC3B-3′UTR-mut luciferase reporter vector, pRL-TK, and NC or miR-204 mimics for 36 h and then harvested for luciferase reporter assay. **C, E** miR-204 mimics or mutants at different doses were co-transfected into 3D4/21 cells with LC3B-3′UTR-wt luciferase reporter vector and pRT-TK for 36 h, and then the activities of luciferase were measured. **D, F** PAMs were transfected with different doses of miR-204 mimics or mutants for 36 h, and the expression of LC3B protein was detected by Western blot. **G** PAMs were transfected with NC or miR-204 mimics for 30 h, followed by lysis of cells and immunoprecipitation with either anti-Ago2 or anti-IgG. RT-qPCR was performed to analyze the presence of LC3B and miR-204 in the cell immunoprecipitates. Results are expressed as means ± standard deviation from three independent experiments. *P* values were analyzed using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

To obtain further evidence that LC3B is directly targeted by miR-204, we performed an RNA-induced silencing complex (RISC) immunoprecipitation assay. PAMs were transfected with miR-204 mimics for 30 h and then cells were harvested to pull down LC3B mRNA and miR-204 with Ago2 monoclonal antibody. As shown in Fig. 3G, LC3B mRNA was significantly enriched in the RISC complex together with miR-204, suggesting that there is a direct interaction between miR-204 and LC3B mRNA. Overall, these results indicate that miR-204 directly targets LC3B and represses its expression.

3.3. Autophagy enhances PRRSV replication

Although previous reports have shown that PRRSV utilizes autophagy to promote self-replication, studies are conducted almost exclusively in Marc-145 cells (a cell line derived from Green Monkey kidney cell) rather than in PAMs (the native host cells of PRRSV) (Chen et al., 2012; Li et al., 2016b; Sun et al., 2012). Thus, we investigated the relationship between autophagy and PRRSV replication in PAMs. PAMs were pretreated with rapamycin, an autophagy activator, for 2 h prior to PRRSV infection. Then, cells were harvested and subjected to Western blot analysis. The results demonstrated that PRRSV infection induced autophagy, and the expression of PRRSV N protein was enhanced when autophagy was induced by rapamycin before viral infection (Fig. 4A). In contrast, the level of PRRSV N protein was downregulated when PAMs were pretreated with 3-methyladenine (3-MA), an autophagy inhibitor (Fig. 4B). These data suggest that induction of autophagy promotes PRRSV replication in PAMs. For further verification, we designed two specific siRNAs targeting LC3B (si-LC3B-1 and si-LC3B-2). As shown in Fig. 4C and D, both siRNAs efficiently knocked down LC3B expression in PAMs at both mRNA and protein levels. Subsequently, we transfected siRNAs into PAMs for 24 h followed by PRRSV infection and then performed RT-qPCR and $TCID_{50}$ assay. Compared to NC, knockdown of LC3B by siRNAs

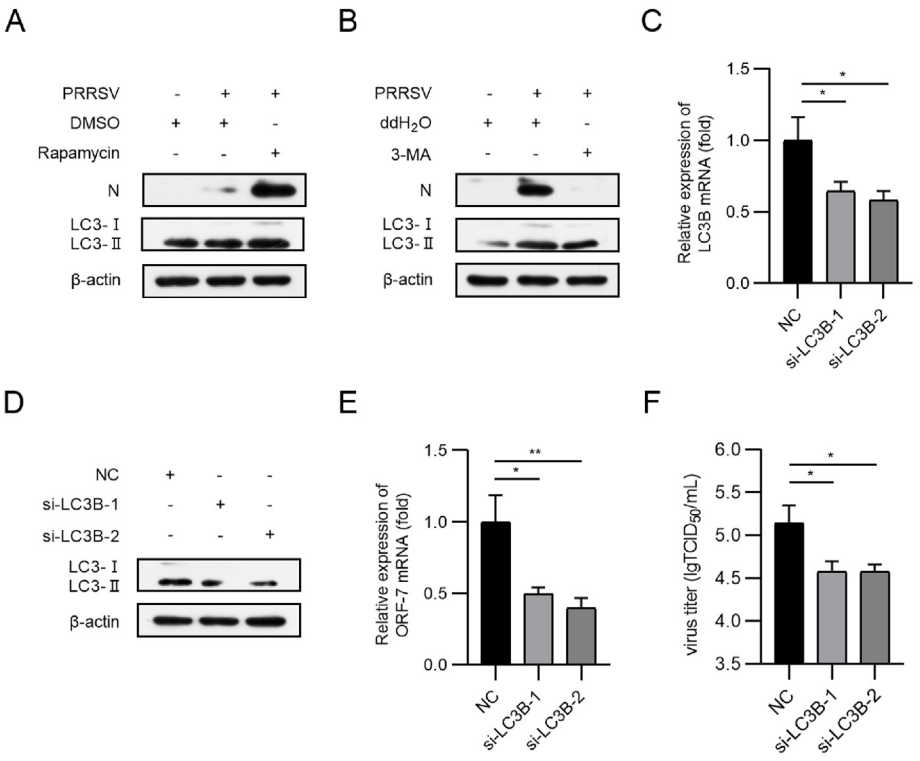

Fig. 4. Induction of cellular autophagy enhances PRRSV replication in PAMs. **A, B** PAMs were pretreated with autophagy activator rapamycin (50 μmol/L) (**A**) or autophagy inhibitor 3-MA (1 mmol/L) (**B**) for 2 h followed by PRRSV infection (MOI = 0.01) for 24 h. Then, the effect of rapamycin, or 3-MA on PRRSV replication was tested by Western blot analysis. **C, D** PAMs were transfected with LC3B-specific siRNAs (60 nmol/L) for 24 h, and then the expression of LC3B was analyzed by RT-qPCR (**C**) or Western blot (**D**). **E, F** PAMs were transfected with LC3B-specific siRNAs (60 nmol/L) for 24 h followed by infection with PRRSV (MOI = 0.01). At 24 h later, cells were harvested to analyze PRRSV ORF7 expression by RT-qPCR (**E**), and the culture supernatant was collected to analyze viral titers (**F**). Results are expressed as means ± standard deviation from three independent experiments. *P* values were calculated using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

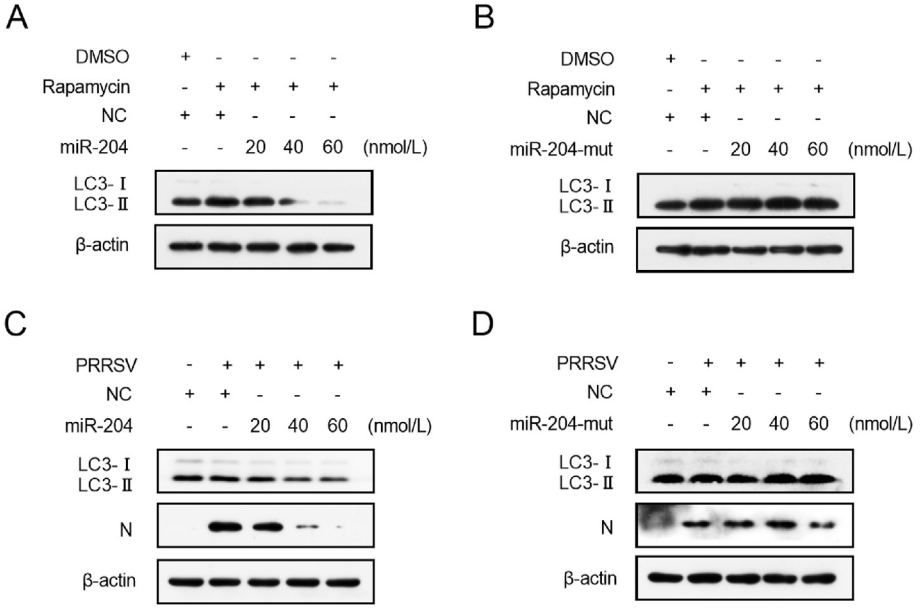

Fig. 5. miR-204 suppresses PRRSV replication by inhibiting autophagy via targeting LC3B. **A, B** PAMs were transfected with miR-204 mimics (**A**) or mutants (**B**) for 24 h followed by treatment with rapamycin for 2 h, and then cells were harvested for Western blot to detect the expression of LC3B protein. **C, D** PAMs were transfected with miR-204 mimics (**C**) or mutants (**D**) for 24 h followed by infection with PRRSV for another 24 h, and then cells were harvested for Western blot analysis to detect the expression of LC3B and PRRSV N protein.

significantly inhibited the expression of PRRSV ORF7 (Fig. 4E). Correspondingly, the viral titers were significantly decreased in LC3B-silenced cells (Fig. 4F). In conclusion, these data demonstrate that PRRSV infection induces autophagy, which in turn facilitates viral replication.

3.4. *miR-204 represses autophagy by targeting LC3B, thus inhibiting PRRSV replication*

Since miR-204 directly targets LC3B, we speculate that miR-204 represses PRRSV replication by inhibiting autophagy. To test this assumption, we first transfected miR-204 mimics into PAMs for 24 h and then treated cells with rapamycin for 2 h. Cells were then harvested for Western blot analysis. As shown in Fig. 5A and B, application of miR-204 mimics had a negative effect on autophagy induced by rapamycin, while miR-204 mutants had no effect. These results imply that miR-204 has an inhibitory effect on autophagy. To explore whether miR-204 inhibits PRRSV replication by interfering with autophagy induced by PRRSV, PAMs were transfected with miR-204 mimics for 24 h followed by PRRSV infection. Then, we observed that the expression of PRRSV N protein and LC3B protein was significantly downregulated in miR-204-transfecting cells in a dose-dependent manner (Fig. 5C). However, this inhibitory phenomenon disappeared when the cells were transfected with miR-204 mutants (Fig. 5D). Taken together, we conclude that miR-204 remarkably inhibits autophagy induced by PRRSV infection via directly targeting LC3B, leading to the inhibition of viral replication.

4. Discussion

In order to cope with viral infection, hosts have often taken a variety of measures to defense themselves, and miRNAs play a non-negligible role in it. Here, we found that host miRNA miR-204 expressed lower in PRRSV target cells (PAMs) had the ability to resist PRRSV replication. Subsequently, we verified that miR-204 directly targeted LC3B, which is necessary for the formation and maturation of autophagosome. Meanwhile, we demonstrated that autophagy induced by rapamycin enhanced PRRSV replication in PAMs, while inhibition of autophagy by 3-MA or specific siRNAs impaired viral replication. Thus, we conclude that miR-204 represses autophagy by targeting LC3B, thereby inhibiting PRRSV replication.

PRRS is a major economic issue for the swine industry worldwide. The annual economic cost of PRRS estimated for U.S. producers is around $664 million, an increase of 18.5% over the past eight years (Derstuganova et al., 2013; Neumann et al., 2005). The latest economic survey in Germany shows that pig farms have experienced a significant loss due to PRRS and its impact on farm profits is −19.1% on average and −41% in the worst cases (Renken et al., 2021). To deal with PRRS, researchers have developed different vaccines. However, the protection provided by current vaccines is limited due to high frequency mutations, gene recombination between different PRRSV lineages, and immunosuppression caused by PRRSV (Montaner-Tarbes et al., 2019). Therefore, to study PRRSV replication-regulations will provide help for the prevention and control of PRRS.

Increasing evidence has documented that host miRNAs participate in modulating PRRSV replication (Liu et al., 2017; Zhang and Feng, 2021). The regulatory mechanisms can be roughly divided into three types according to their targets. First, miRNAs directly target the genome of PRRSV. miR-23, miR-505 and miR-378 effectively suppress PRRSV replication by directly targeting viral RNAs (Zhang et al., 2014). Secondly, miRNAs can target signaling pathways especially involved in host antiviral immune response. For instance, miR-150 upregulated by PRRSV infection via activating the PKC/JNK/c-Jun pathway can impair PRRSV replication by targeting SOCS1, a negative regulator of JAK/STAT signaling pathway (Li et al., 2022). In contrast, miR-30c inhibits the function of type I interferon by targeting JAK1 and IFNAR2 expression and then promotes PRRSV replication (Liu et al., 2018; Zhang et al., 2016). Thirdly, miRNAs can target viral receptors to block PRRSV entry. The PRRSV receptors, including CD163, CD151 and MYH9, appear to be targeted by miR-181, miR-506, and let-7f-5p, respectively (Guo et al., 2013; Li et al., 2016a; Wu et al., 2014). In the present study, we find that miR-204 strongly suppresses PRRSV replication by inhibiting autophagy via directly targeting LC3B.

To date, an increasing number of studies have revealed the direct interaction between autophagy and infection processes of multiple pathogens. As an important innate antiviral response, autophagy can

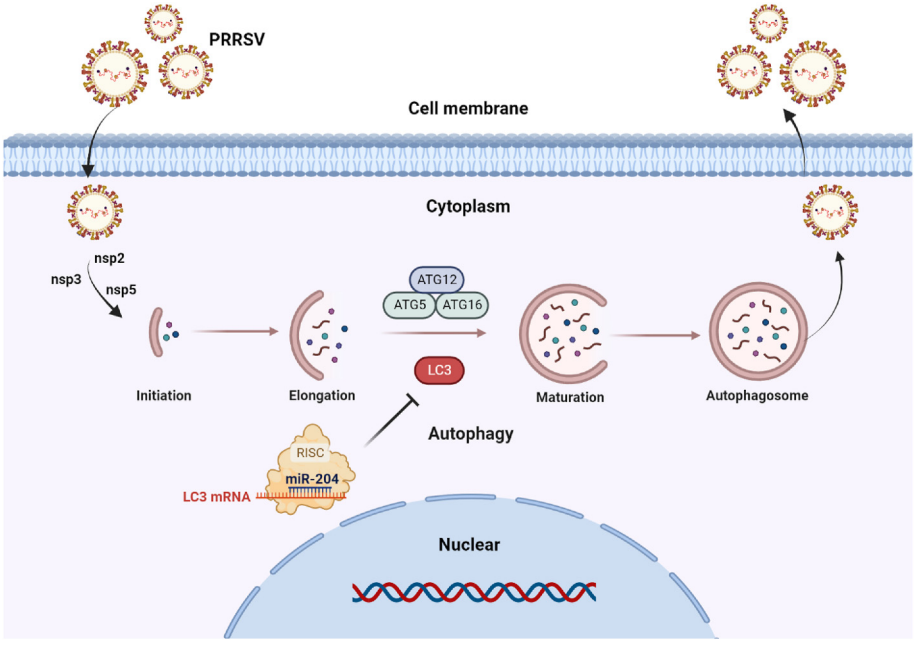

Fig. 6. Model for the mechanisms by which miR-204 suppresses PRRSV replication. PRRSV infection induces autophagy, which is utilized by virus to promote its replication. miR-204 directly targets LC3B to suppress autophagy, leading to the inhibition of PRRSV replication. Figure was created with BioRender (https://biorender.com).

degrade viral components, viral particles or even host factors required for viral replication to resist viruses, such as Sindbis virus and Human immunodeficiency virus 1 (Orvedahl et al., 2010; Ribeiro et al., 2016). However, some viruses have evolved various strategies to escape the clearance by autophagy, and even exploit autophagy to ensure their survival or replication in hosts. For example, hepatitis C virus (HCV) NS4B induces Rubicon expression in the early stage of viral infection to inhibit the maturation of autophagosomes, leading to the accumulation of autophagosomes in support of HCV replication (Wang et al., 2015). miRNAs have been shown to play an important role in the interaction between viral replication and autophagy. For instance, miR-146a-5p, which is upregulated by hepatitis B virus (HBV) infection, induces autophagy via the XIAP-MDM2/p53 pathway, leading to HBV propagation (Fu et al., 2019). On the contrary, miR-505 inhibits autophagy activation by reducing HMGB1 expression, and ultimately inhibits the replication of Borna disease virus 1 (BDV1) (Guo et al., 2022).

Over the past few years, more and more attention has been paid to the effect of autophagy on PRRSV replication. PRRSV infection induces incomplete autophagy, and the fusion of intracellular lysosomes and autophagosomes is limited, resulting in the accumulation of autophagosomes and ultimately serving as replication sites for PRRSV (Sun et al., 2012). Moreover, NDRG1 expression is downregulated by PRRSV infection, which induces autophagy, changes cellular lipid metabolism, and ultimately promotes PRRSV replication (Wang et al., 2019). A recent report has found that PRRSV infection induces endoplasmic reticulum stress, opens store operated calcium entry (SOCE) channel, disrupts host calcium homeostasis, and utilizes autophagy via CaMKII-AMPK-mTOR signaling to promote viral replication (Diao et al., 2023). In the present study, treatment with rapamycin (activator of autophagy) prior to viral infection led to a significant upregulation of LC3B and PRRSV N proteins in PAMs. Conversely, 3-MA (inhibitor of autophagy) reduced the expression of LC3B as well as PRRSV N protein. These results are consistent with previous studies that autophagy is induced and utilized by PRRSV for its replication (Fig. 4). Interestingly, we found that miR-204 was highly expressed in PPMs, the non-permissive cells to PRRSV, but extremely lower in PAMs, the target cells of PRRSV. Moreover, we found that the expression of miR-204 was not regulated by PRRSV infection. Hence, it is reasonable to assume that it is beneficial for PRRSV to hijack autophagy to promote its proliferation when miR-204 is kept at an extremely lower level in PAMs during PRRSV infection.

LC3B, the most studied and best understood protein of LC3/GABARAP family, is essential for the execution of autophagy (Schaaf et al., 2016). Here, we revealed a previously unreported relationship between LC3B, PRRSV, and miR-204 in the context of viral infection. Bioinformatics analysis showed that LC3B could be a direct target of miR-204, which was later confirmed by luciferase reporter assay and CLIP assay. During viral infection, we observed that overexpression of miR-204 significantly reduced the protein levels of LC3B as well as PRRSV N, while miR-204 mutants had no effect. Based on these results, we conclude that miR-204 suppresses autophagy by downregulating LC3B, thus impairing PRRSV replication.

5. Conclusions

Overall, our findings prove that miR-204 is a negative regulator of PRRSV replication by regulating LC3B-mediated autophagy, which will help us for the development of novel antiviral strategies against PRRSV infection (Fig. 6).

Data availability

All the data generated during the current study are included in the manuscript. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This article does not contain any studies with human or animal subjects performed by any authors.

Author contributions

Yao Yao: conceptualization, formal analysis, data curation, investigation, writing- original draft, methodology. Sihan Li: methodology. Yingqi Zhu: methodology. Yangyang Xu: methodology. Siyuan Hao: methodology. Shuyuan Guo: methodology. Wen-hai Feng: funding acquisition, resources, conceptualization, supervision, project administration, writing-review and editing. All authors have read and agreed to the version of the manuscript.

Conflict of interest

Prof. Wenhai Feng is an Editorial Board member for *Virologica Sinica* and was not involved in the editorial review or the decision to publish this article. The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2023.07.004.

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