Veterinary Microbiology 228 (2019) 147–156

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

High level expression of ISG12(1) promotes cell apoptosis via mitochondrial-dependent pathway and so as to hinder Newcastle disease virus replication

Xiaoqin Li[1], Yanqing Jia[1], Haijin Liu, Xiangwei Wang, Zhili Chu, Ruochen Hu, Juan Ren, Sa Xiao, Shuxia Zhang, Xinglong Wang*, Zengqi Yang*

College of Veterinary Medicine, Northwest A&F University, Yangling 712100, China

ARTICLE INFO

Keywords:
ISG12(1)
Newcastle disease virus (NDV)
Antiviral
Apoptosis

ABSTRACT

Newcastle disease (ND), caused by virulent Newcastle disease virus (NDV), poses a considerable risk for the poultry industry. A comprehensive understanding of the interaction between NDV and its host is therefore critical for control of this disease. Previously, we found that chicken ISG12(1) was among the significantly upregulated interferon-stimulated genes (ISGs) in embryos and the bursa of Fabricius of chickens infected by NDV, based on transcriptome sequencing. However, its antiviral effects and function were poorly understood. In this study, we aimed to determine the effects of chicken ISG12(1) on NDV replication. First, we confirmed that NDV infection stimulated high level expression of chicken ISG12(1) in vivo and in vitro based on RT-qPCR. Next, through overexpression and knockdown experiments, the antiviral activity of ISG12(1) was investigated. As expected, this protein was found to hinder NDV replication. In addition, we showed that ISG12(1) localized to the mitochondria; promoted the redistribution of Bax, a proapoptotic protein causing irreversible loss of mitochondrial function, from the cytoplasm to the mitochondria; and therefore induced cell apoptosis. In conclusion, elucidation of the role of chicken ISG12(1) in combatting NDV infection contributes to our understanding of the responses of poultry to viruses and may facilitate the generation of more efficient vaccines to control ND.

1. Introduction

Newcastle disease virus (NDV), also known as avian paramyxovirus 1 (APMV-1), belongs to the avian-infecting genus *Avulavirus* of the Paramyxoviridae family (Amarasinghe et al., 2018). Its genome consists of a negative-sense, single-stranded RNA encoding six structural proteins—nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase (L)—as well as two nonstructural proteins, V and W (Alexander, 2000; Krishnamurthy, 1998). Based on mean death time (MDT) and clinical signs in chickens, NDVs are divided into lentogenic, mesogenic, and velogenic stains (Dortmans et al., 2011; Dimitrov et al., 2016). The virulent strain is the etiologic agent of Newcastle disease (ND), which poses a considerable risk for the poultry industry. Despite the use of vaccines to prevent this disease, ND frequently outbreaks in many countries (Zhu et al., 2016; Dimitrov et al., 2017), indicating that vaccination alone is insufficient to control ND under some conditions. NDV HN recognizes receptors on host cells to initiate the infection process, accompanied by strong immune responses that block viral transcription and replication, among others (Ganar et al., 2014). Therefore, understanding the host immune response to NDV infection is important for ND prevention.

Interactions between intracellular pathogen recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) activate a series of intracellular signal transduction pathways that increase the production of numerous factors such as interferons (IFNs), interleukins, and cytokines (Takeuchi et al., 2009; Takaoka et al., 2006). Among these, IFNs are critical for host defense against viruses (Haller et al., 2009). These proteins, secreted from infected cells, bind to specific cell surface receptors and stimulate the Janus kinase–signal transducer and

* Corresponding author.
E-mail addresses: L18584890329@163.com (X. Li), yqjia1987@163.com (Y. Jia), liuhaijin@nwafu.edu.cn (H. Liu), wxw2014hc@163.com (X. Wang), zhilichu@nwafu.edu.cn (Z. Chu), 18829349275@163.com (R. Hu), m15591051263@163.com (J. Ren), saxiao@nwafu.edu.cn (S. Xiao), zhangshuxia316@163.com (S. Zhang), wxlong@nwsuaf.edu.cn (X. Wang), yzq8162@163.com (Z. Yang).
[1] Contributed equally.

https://doi.org/10.1016/j.vetmic.2018.11.017
Received 12 September 2018; Received in revised form 14 November 2018; Accepted 16 November 2018

activator of transcription (JAK–STAT) signaling pathway (Liu et al., 2011; Borden et al., 2007; Pestka et al., 2004). This activates hundreds of IFN-stimulated genes (ISGs) to establish an antiviral state (MacMicking, 2012; de Veer et al., 2001). However, only a few of these ISGs have been extensively studied in terms of anti-NDV activity. For example, PKR is activated by NDV dsRNA to impair viral replication via eIF2α phosphorylation in HeLa cells (Zhang et al., 2014).

ISG12 genes, first identified in the estradiol-treated human breast epithelial cell line MCF7, are induced by IFN stimulation and widely expressed in various species (Rasmussen et al., 1993; Cheriyath et al., 2011). There are four ISGs (6–16, ISG12a, ISG12b, and ISG12c) in humans, three (ISG12a, ISG12b1, and ISG12b2) in mice, and two (ISG12-1 and ISG12-2) in chickens (Parker et al., 2004). Previous studies have shown that ISG12 genes can disturb viral replication (Lu et al., 2011; Qi et al., 2015; Meyer et al., 2015; Yang et al., 2014; Chen et al., 2017); for example, ISG12 was suggested to have antiviral effects, protecting neonatal mice from Sindbis virus-induced death (Labrada et al., 2002) and potentially protecting against infection by hepatitis C virus (HCV; Itsui et al., 2006) and multiple neurotropic viruses (Cho et al., 2013). A recent report indicated that ISG12a specifically targets HCV NS5 A for degradation to inhibit HCV replication via apoptosis (Xue et al., 2016). However, whether chicken ISG12(1) hinders NDV replication and the associated pathways is unclear.

In our previous study, RNA-seq was performed to analyze the transcriptomes of chicken tissues infected by NDV (Jia et al., 2018). Chicken *ISG12(1)* was one of the most highly upregulated ISGs in embryos and the bursa of Fabricius tissues of specific-pathogen-free (SPF) chickens infected, suggesting that it may play a pivotal role in NDV infection (Jia et al., 2018). Here, we investigated the function of chicken ISG12(1) and the possible associated mechanisms in against NDV infection using overexpression and knockout experiments. Our results indicate that ISG12(1) is a mitochondrial protein and reduces NDV replication by mediating intrinsic cell apoptosis process.

2. Materials and methods

2.1. Ethics statement

All studies and regulatory licenses were approved by the Committee for the Ethics of Animal Care and Experiments of Northwest A&F University (Approval number: 2015ZX08008016-016).

2.2. Cells and virus

Chicken embryo fibroblasts (CEFs) were prepared from 10-day-old SPF chicken embryos. In brief, eggshells were disinfected and opened, and the head, extremities, and viscera were removed. Tissues were washed three times with phosphate-buffered solution (PBS). The remaining muscle tissues were cut with sterile scissors, washed three times with PBS, digested in 0.25% trypsin using a magnetic stirrer for 15 min, and centrifuged. The precipitate was re-suspended in PBS, filtered through a 3-tier sieve to collect CEFs. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 incubator.

The velogenic class II genotype IX NDV strain, F48E9, the lentogenic class II genotype II NDV strain, La Sota, and the mesogenic class I NDV strain, CS2, were maintained in our laboratory and propagated in the allantoic cavities of 10-day-old SPF embryonated chicken eggs. Two days after amplification, allantoic fluid was collected and filtered, and viral titers were determined by hemagglutinin (HA) testing. A multiplicity of infection (MOI) of 0.1 was used for infection in this study.

2.3. Library construction and deep sequencing

Library construction and deep sequencing were performed according to the methods from Jia et al. (2018). In brief, the F48E9 or La Sota strain was injected into the allantoic cavities of 10-day-old chicken embryos at a dosage of 10^4 plaque-forming units (PFU)/egg. The same volume of PBS was injected as a negative control. Visceral tissues were collected from each group at 36 h post-infection (hpi) and sent to Novogene for sequencing (Wuhan, China).

In another experiment, 4-week-old SPF chickens were infected with F48E9 (2×10^4 PFU/chicken), and transcriptome sequencing was performed by using the bursa of Fabricius tissues collected at 48, 72, and 96 hpi.

2.4. Plasmid construction and transfection

Primers for cloning of *ISG12(1)* were designed with a Flag tag at the C-terminus according to the published *Gallus gallus ISG12(1)* sequence (BN000222) as follows: F: 5′-GGAATTCATGAATCCGAACATCATC-3′, R: 5′-GAAGATCTTTACTTATCGTCGTCATCCTTGTAATCAGGGATTTTCAAACC

3′) (The underlined sequence represents Flag tag sequence). Chicken *ISG12(1)* cDNA was synthetized, amplified and then inserted into the *Eco*RI/BgIII (TaKaRa, Dalian, China) sites in pCAGGS to construct pCAGGS-ISG12(1)-Flag (pCA-ISG12(1)-Flag). Successful insertion was confirmed by sequencing (TSINGKE, Xian, China). Next, 5×10^5 DF-1 cells/well were cultured in a 24-well plate overnight before being transfected with 1 μg plasmid using 2 μL TurboFect Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) to overexpress *ISG12(1)*. RNA interference was used to knock down *ISG12(1)* expression. The siRNA sequences were designed and synthesized by the Sangon Company (Shanghai, China) as follows: ISG12(1)-chicken-118 (si118) sense: 5′-GGUUCCAUAGCAGCCAAGATT-3′, antisense: 5′-UCUUGGCUGCUAUGGAACCTT-3′; ISG12(1)-chicken-222 (si222) sense: 5′-GCUCUCUUUCGUUGCCAAATT-3′, antisense: 5′-UUUGGCAACGAAAGAGAGCTT-3′; ISG12(1)-chicken-11 (si11), sense: 5′-ACAUCAUCAAAGCCGCCAUTT-3′, antisense: 5′-AUGGCGGCUUUGAUGAUGUTT-3′; negative control (NC) sense: 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense: 5′-ACGUGACACGUUCGGAGAATT-3′. For knockdown, 20 pmol siRNA was transfected into DF-1 cells using 2 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 36 and 48 h after transfection, ISG12(1) overexpression or knockdown was confirmed by western blotting.

2.5. RNA extraction and real-time quantitative PCR (RT-qPCR)

DF-1 cells were plated at 5×10^5 per well in a 24-well plate and incubated for 24 h.

Cells were then transfected with pCA-ISG12(1)-Flag or siRNA, as described previously. After 24 h, cells were subsequently infected with NDV; after an additional 24 h, cells were harvested for further experiments. The relative expression levels of the genes encoding ISG12(1), NDV M, Bax, Bak, Bcl-2, Bcl-xl, cytochrome c (Cyt c), caspase-3, and caspase-9 were quantified by RT-qPCR using the specific primers listed in Table 1. Total cellular RNA was extracted using TRIzol reagent (Invitrogen), and cDNA was synthesized via reverse transcription using the PrimeScript RT reagent kit (Takara) according to the manufacturer's protocol. The RT-qPCR reactions were carried out in 96-well blocks using 2× qPCR Master Mixture (DiNing, Beijing, China) with the following reaction conditions: 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 30 s. The 28S rRNA housekeeping gene was used as a reference to normalize the expression levels of target genes, and the relative gene expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method.

2.6. Western blotting

Cells were washed with PBS and lysed on ice for 15 min using radioimmunoprecipitation buffer with the proteinase inhibitor PMSF.

Table 1
Primers used for real-time RT-qPCR analysis.

Primers	Sequence (5′–3′)
28S	qF: GGTATGGGCCCGACGCT
	qR: CCGATGCCGACGCTCAT
NDV M	qF: CCGATCGTCCTACAAGACACAG
	qR: GGACGCTTCCTAGGCAGAGCAT
ISG12(1)	qF:GGGTTCCATAGCAGCCAAG
	qR:CAACGAAAGAGAGCCCCGC
caspase3	qF:CCATGGCGATGAAGGACTCT
	qR:CCCGCTAGACTTCTGCACTT
caspase9	qF:GCTTGTCCATCCCAGTCCAA
	qR:CAGTCTGTGGTCGCTCTTGT
Bcl-2	qF:CTTCCGTGATGGGGTCAACT
	qR:AGGTACTCGGTCATCCAGGT
Bcl-xl	qF:GCATCGTGGCTTTCTTCTCC
	qR:CAGTACCCGCATCTCCTTGT
Bax	qF:GGTGACAGGGATCGTCACAG
	qR:TAGGCCAGGAACAGGGTGAA
Bak	qF:CTGTTCGCTTCCTTCCCCTG
	qR:TTGCAGAGATGCTGTGGGAC
Cyt c	qF:AGGCAAGCACAAGACTGGA
	qR:CTGACTATCACCAAGAACCACC

Protein samples were then boiled for 10 min. Whole-cell lysates of a fixed quantity (20 μg/lane) were separated by 15% SDS-PAGE, followed by transfer to 0.22-μm polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 10% skim milk at 4 °C overnight. Primary and secondary antibodies were incubated for 4 h and 60 min, respectively, at room temperature. The following polyclonal antibodies were used: anti-Flag (1:1000, Solarbio, Beijing, China), anti-HN (1:500, our lab), anti-Bax, anti-caspase-3, anti-Bcl-2 (1:500, Bioss, Beijing, China), anti-β-tubulin, and HRP-conjugated goat anti-mouse/rabbit IgG as a secondary antibody (1:3000, Abcam, Cambridge, MA, USA). Antibodies were diluted in 1% skim milk. Immunoreactive bands were detected using an ECL peroxidase substrate (Millipore).

2.7. Plaque assays

Viral titers were measured by plaque assay, as previously (Chu et al., 2018). In brief, 8×10^4 BHK-21 cells were seeded in 24-well plates 24 h prior to infection. Cells were washed twice with PBS, and 300 μL viral suspension was added and incubated for 1 h. At 1 hpi, supernatants were replaced with RPMI1640 containing 2% FBS and 1% methyl cellulose (Solarbio, Beijing, China), and cells were incubated for an additional 3–4 days. Next, cells were fixed with 5% formaldehyde in PBS and stained with 1% methylene blue. Plaques formed were checked and counted, with each plaque corresponding to one viral particle.

2.8. Cell apoptosis detection by flow cytometry

The effects of ISG12(1) on cell apoptosis were detected using an Annexin V-FITC/PI Apoptosis Detection Kit (CWBIO, Beijing, China) according to the manufacturer's protocol. Briefly, cells were collected with trypsin and re-suspended in 250 μL Annexin V-FITC binding buffer 24 h after transfection. Then, 5 μL Annexin V-FITC and 10 μL propidium iodide (PI) were added, and the cells were incubated for 15 min at room temperature in dark. Cells were analyzed using a FACSAriaT flow cytometer (BD Biosciences, USA). Percentages of early apoptotic cells (Annexin V + /PI −), late apoptotic or necrotic cells (Annexin V + /PI +), and viable cells (Annexin V − /PI −) were determined using FlowJo software.

2.9. Confocal microscopic analysis subcellular localization of target proteins

For detection subcellular localization of ISG12(1), 1×10^4 DF-1 cells were seeded on glass coverslips in 35-mm cell culture dishes and cultured overnight. Cells were then transfected with pCAGGS (Vec) or pCA-ISG12(1)-Flag and cultured for an additional 24 h. Supernatants were removed, and cells were incubated with 200 nM Mito-Tracker Red CMXRos (Yeasen, China) for 45 min at 37 °C. Cells were fixed in 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% TritonX-100 for 10 min at room temperature. Cells were blocked with 1% BSA at 4 °C for 6 h, incubated with anti-Flag polyclonal antibody (1:500, Solarbio) at 4 °C overnight, and then incubated with goat anti-mouse IgG H&L (Alexa Fluor® 488) antibody (1:1000, Abcam) at 37 °C for 1 h. Hoechst 33342 (1:200, Beyotime, Shanghai, China) was added to stain nuclei. Each step was preceded by three PBS washes. Finally, laser scanning confocal microscopy was used to observe fluorescence.

For detection distribution of Bax, cells were transfected with Vec or pCA-ISG12(1)-Flag. After 24 h, supernatants were removed, and cells were incubated with 200 nM Mito-Tracker Red CMXRos (Yeasen) for 45 min at 37 °C. Cells were fixed, permeabilized, and blocked, as described above, and then incubated with rabbit anti-Bax polyclonal antibody (1:500, Bioss) overnight at 4 °C. Then, cells were washed three more times with PBS and incubated with goat anti-rabbit IgG (Alexa Fluor® 488) antibody. Images were captured by confocal microscopy.

To detect co-localization of Bax and ISG12(1), cells were transfected with pCA-ISG12(1)-Flag. After 24 h, cells were directly fixed, permeabilized, and blocked, and then exposed to mouse anti-Flag polyclonal antibody (1:500, Solarbio) and rabbit anti-Bax polyclonal antibody (1:500, Bioss) overnight at 4 °C. Cells were washed three more times with PBS and incubated with goat anti-rabbit IgG (Alexa Fluor® 488) antibody and goat anti-mouse IgG H&L (Alexa Fluor® 594) (1:1000, Abcam) at 37 °C for 1 h. Hoechst 33342 (1:200, Beyotime) was also added to stain nuclei.

2.10. Statistical analysis

All data are the results of at least three independent experiments. Statistical analyses were performed using Student's *t*-tests as implemented in GraphPad Prism 5.0 software (San Diego, CA, USA). A *P*-value < 0.05 was used to indicate statistical significance.

3. Results

3.1. Expression of ISG12(1) after NDV infection in vivo and in vitro

To confirm the up-regulation of ISG12(1) upon NDV infection, the transcriptomes of infected chicken embryos and the bursa of Fabricius tissues were analyzed., The expression levels of ISG12(1) mRNA significantly increased ($p < 0.05$)after NDV infection (Fig. 1A). The finding was further validated by RT-qPCR. In chicken embryos and the bursa of Fabricius tissues, NDV infection increased mRNA levels of ISG12(1) mRNA by up to 450- and 150-fold, respectively, compared to the controls (Fig. 1B and C). We also determined the dynamics of ISG12(1) mRNA expression in CEFs and DF-1 cells after infection with different pathotypes NDV(Fig. 1D and E). In all treated groups, ISG12(1) mRNA expression levels were up-regulated after NDV infection compared to therefrom mock-infected groups. In addition, Expression levels of ISG12(1) reached their peak at 24 hpi. These results suggest that NDV infection induces ISG12(1) expression, indicating that it may be related with host response against NDV infection.

3.2. High level expression of ISG12(1) reduced NDV replication

To examine the effect of ISG12(1) on NDV replication, pCA-ISG12(1)-Flag was transfected into DF-1 cells, followed by NDV infection. At 24 h after transfection, ISG12(1) overexpression was confirmed by western blotting and RT-qPCR. Due to the lack of an ISG12(1)-specific antibody, an anti-Flag antibody was used to detect the Flag tag fused to the C-terminus of ISG12(1). Using the anti-Flag antibody, we

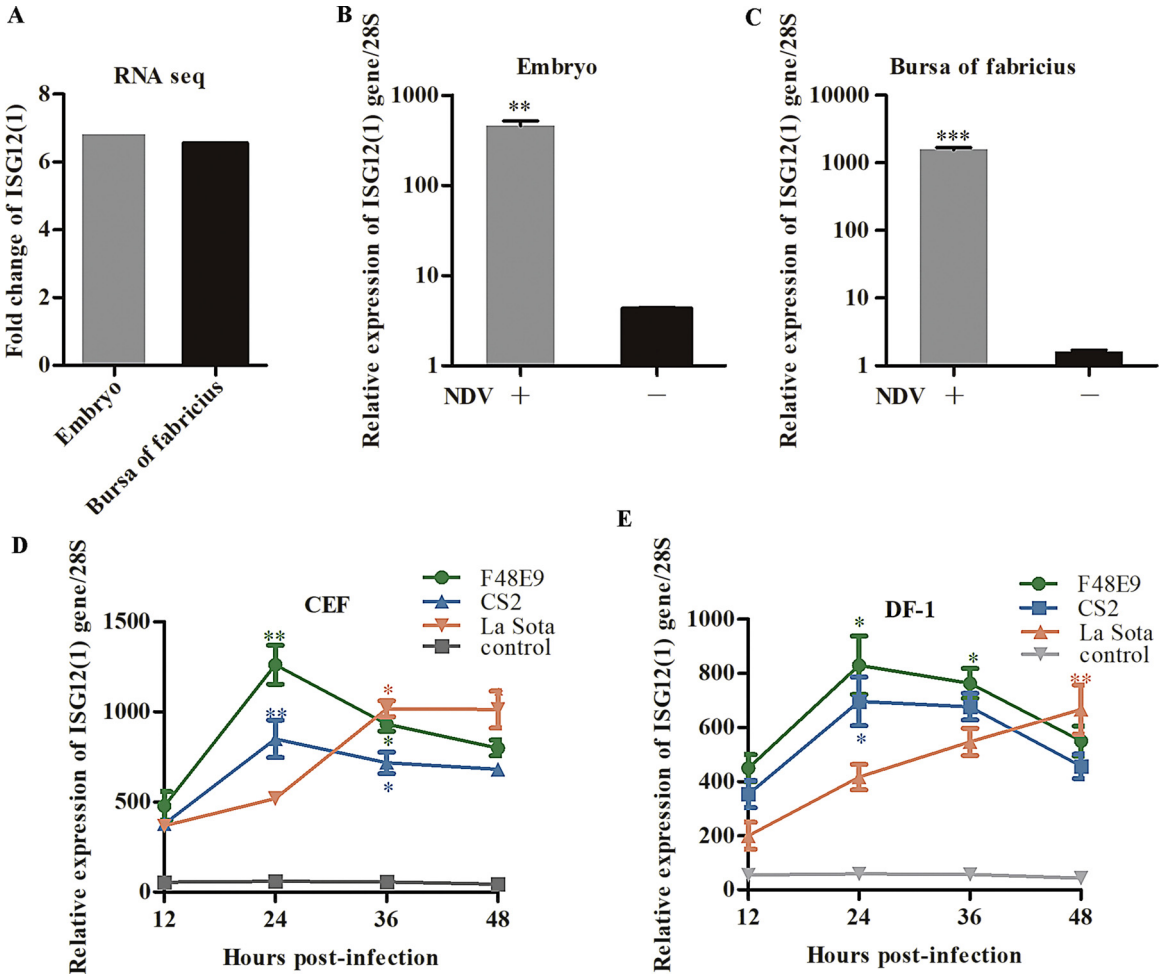

Fig. 1. NDV infection induced ISG12(1) overexpression in vivo and in vitro.
Expression of ISG12(1) was detected and analyzed by RNA-seq (A) and validated by real-time quantitative PCR (RT-qPCR) in specific-pathogen-free (SPF) chicken embryos (B) and the bursa of Fabricius (C) of SPF chickens treated with PBS or infected with NDV for 24 h. ISG12(1) mRNA expression in CEFs (D) and DF-1 cells (E) was assessed after NDV (F48E9\CS2\La sota) infection at different times by RT-qPCR. Data are showed as means ± SDs of three independent experiments.

confirmed the expression of ISG12(1) protein (approximately 11 kDa) in cells transfected with pCA-ISG12(1)-Flag, whereas no targer protein expression were detected in cells transfected with empty vector (Vec, Fig. 2A). In addition, mRNA expression of *ISG12(1)* increased by 400-fold compared to that in control cells (Fig. 2B). Moreover, overexpression resulted in reduction of NDV replication. As shown in Fig. 2C, viral loads represented by M gene level of NDV detected by RT-qPCR were significant ($p < 0.05$) decreased after infection. Additionally, based on plaque assay results, there were fewer infective viral particles in the supernatants from cells transfected with pCA-ISG12(1)-Flag than from control (Fig. 2D). Although virus proliferations were observed, overexpression ISG12(1) significant inhibited NDV replication compared with the control. The viral titers in the supernatants of cells transfected with pCA-ISG12(1)-Flag were approximately 3×10^5 and 6×10^5 PFU/mL at 12 and 24 hpi, respectively; these were significantly ($p < 0.05$) lower than the control with titers of 7×10^5 PFU/mL and 12×10^5 PFU/mL, respectively (Fig. 2E). High level expression of ISG12(1) blocked viral replication also demonstrated by analysis protein HN expression (Fig. 2F).

Accordingly, we speculated that ISG12(1) negatively regulation NDV replication, therefore, knockdown ISG12(1) expression would promote NDV replication. Three different siRNAs were used to down-regulation ISG12(1) expression and as we found, siRNA si118 was optimal (Fig. 2G). Hence, si118 was transfected into DF-1 cells, and which were then infected with NDV. Based on detection RNA, protein, and viral particle levels, we proved that down-regulation ISG12(1) expression improved NDV amplification in cells, as expected (Fig. 2H–K). Taken together, these results suggest that NDV infection stimulates up-regulate the expression of ISG12(1), which is an antiviral molecule.

3.3. ISG12(1) enhances NDV-induced cell apoptosis in DF-1 cells

The above results indicate that chicken ISG12(1) hinders NDV growth, but the associated mechanism remained unknown. To explore the mechanism, DF-1 cells overexpressing ISG12(1) were stained with Annexin V and PI, and then analyzed by flow cytometry. Interestingly, we found that high level expression ISG12(1) significantly enhanced cell apoptosis response in NDV infected DF-1 cells. The percentage of the cells in early apoptotic stage (Annexin V+) was 29.1% in DF-1 cells overexpressing ISG12(1), but the percentage of the control cells was only 3.46%. In addition, ISG12(1) transfection resulted in a percentage of 12.1% cells in late apoptotic stage (Annexin V+/PI+). Furthermore, the ratios of the cells, transfected with pCA-ISG12(1)-Flag- and Vec, in late apoptotic stage were 44.0% and 14.6%, respectively, after NDV infection (Fig. 3A and B). In contrast, siRNA-mediated down-regulation of ISG12(1) reduced cell apoptosis in infected DF-1 cells, even blocking cell apoptosis caused by NDV infection (Fig. 3C and D). These results identified that anti-NDV activity of ISG12(1) is related with cell apoptosis response.

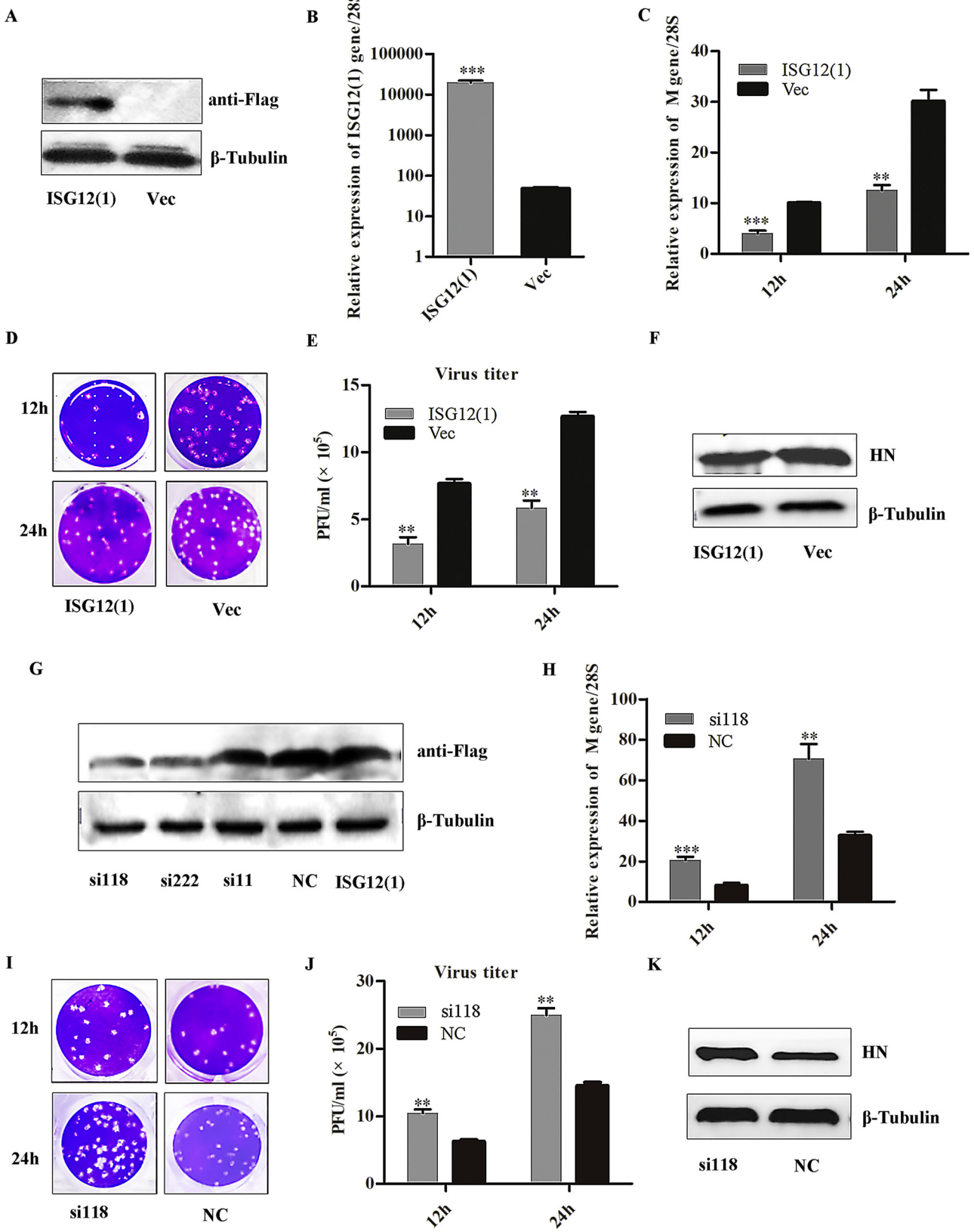

Fig. 2. ISG12(1) hinders Newcastle disease virus (NDV) replication in DF-1 cells.
DF-1 cells were transfected with pCA-ISG12(1)-Flag or pCAGGS (Vec). High level expression of ISG12(1) in DF-1 cells was confirmed by western blotting (A) and RT-qPCR (B). DF-1 cells were transfected with pCA-ISG12(1)-Flag or pCAGGS (Vec) and then infected with NDV at a MOI of 0.1. Viral quantities were assessed at indicated time points by RT-qPCR (C), plaque titration (D,E) and western blotting (F) at 24 h post-infection. DF-1 cells were co-transfected with a pCA-ISG12(1)-Flag expression plasmid and non-targeting control siRNA (NC) or ISG12(1)-targeting siRNAs, followed by western blotting analysis using an anti-Flag antibody. The efficiencies of the three siRNAs were compared by western blotting (G). DF-1 cells were transfected with siRNAs, si118 or non-targeting control siRNA (NC) and infected with NDV at a MOI of 0.1. Viral quantities were then determined (H–K). Data are presented as means ± SDs of three independent experiments.

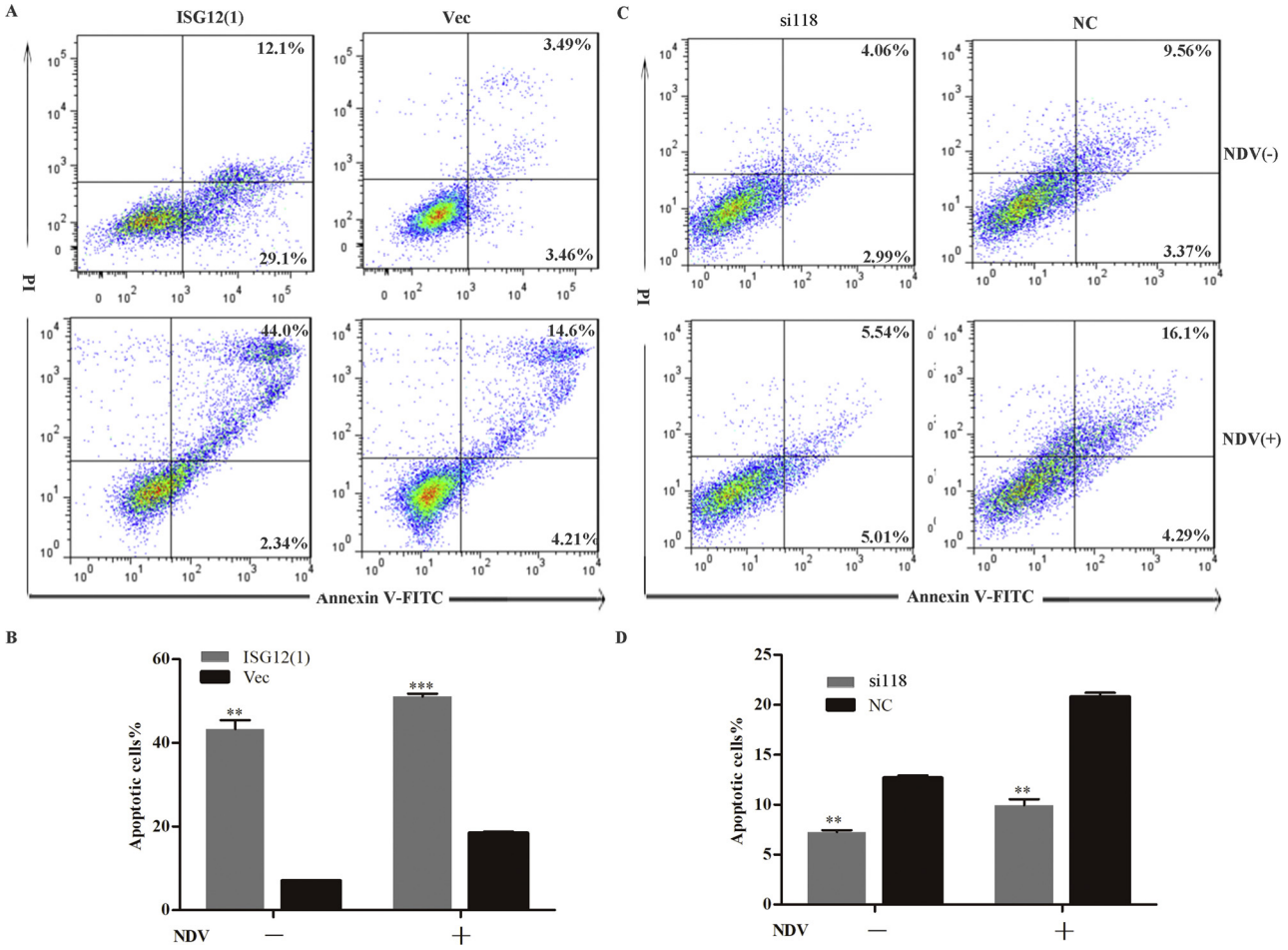

Fig. 3. ISG12(1) promotes cell apoptosis of DF-1 cells.
Cell apoptosis of DF-1 cells transfected with pCA-ISG12(1)-Flag or pCAGGS (Vec) was detected after AnnexinV-FITC/propidium iodide (PI) labeling using flow cytometry following Newcastle disease virus (NDV) infection (A). The percentages of apoptotic cells (positive for both Annexin V and PI staining) were analyzed (B). DF-1 cells were transfected with non-targeting control siRNA (NC) or ISG12(1)-targeting siRNA (si118) and infected with NDV at a MOI of 0.1; apoptotic cells were detected by flow cytometry at 24 h post-infection (C). Apoptotic cells were analyzed as that done in B (D). Data are presented as means ± SDs of three independent experiments.

3.4. ISG12(1) promotes NDV-induced DF-1 cell apoptosis via mitochondria-related cell apoptosis pathway

To further identify the apoptotic pathway induced by ISG12(1), we examined subcellular localization of ISG12(1) by indirect immunofluorescence and laser confocal microscopy. The results indicated that ISG12(1) localized to the mitochondria (Fig. 4A). In addition, overexpression ISG12(1) resulted in translocation of Bax from the cytoplasm to the mitochondria (Fig. 4B), represented the co-localization of Bax and ISG12(1) in the mitochondria (Fig. 4C).

Overexpression ISG12(1) also elevated Bax expression according western blotting result (Fig. 5A). In addition, overexpression ISG12(1) resulted in various regulation of other markers related to the mitochondrial apoptosis pathway, such as the down-regulation of the anti-apoptotic protein Bcl-2 and up-regulation of the pro-apoptotic protein cleaved caspase-3 (Fig. 5A). In addition, at mRNA level, the genes encoding the pro-apoptotic factors Bax, Bak, Cyt c, caspase-3, and caspase-9 were significantly up-regulated ($p < 0.05$), while those encoding the anti-apoptotic markers Bcl-2 and Bcl-xl were significantly down-regulated in cells overexpressing ISG12(1), as compared to control (Fig. 5C). Moreover, this phenomenon was enhanced during NDV infection (Fig. 5A and D). In contrast, knockdown ISG12(1) expression abolished this regulatory effect; specifically, the expression of anti-apoptosis factors (such as Bcl-2) was enhanced, whereas the expression of the pro-apoptosis factors (such as Bax) were declined (Fig. 5B and E). Even in case of NDV infection failed to up-regulate the expression of these pro-apoptotic markers in DF-cells down-regulation ISG12(1) with siRNA, si118 (Fig. 5B and F). Collectively, these data demonstrated that ISG12(1) reduces NDV propagation via activation mitochondria-related apoptosis pathway (Fig. 6).

4. Discussion

Understanding the host response to NDV is critical for generating improved solutions for combatting this devastating disease. Multiple studies have shown that NDV infection stimulates the expression of various genes to induce a host immune response (Liu et al., 2012; Kaiser et al., 2016; Liu et al., 2018; Deist et al., 2017). In previous study, we found that the expression of ISG12(1) was substantially increased in embryos and the bursa of Fabricius tissues of SPF chickens using RNA-seq. Furthermore, after infection of CEFs or DF-1 cells with NDV strains of varying virulence, ISG12(1) was significantly up-regulated. In fact, virulence NDV strain induced higher level of ISG12(1) expression. Virus infection triggers IFN responses that activate downstream IFN signaling pathways, which then activate hundreds of ISGs, including ISG12 (Randall et al., 2008; Rue et al., 2011; Schneider et al., 2014; Kang et al., 2016). Similarly, NDV infection induced IFNs generation (Hu et al., 2015; Xiang et al., 2018). However, whether IFNs involved in the

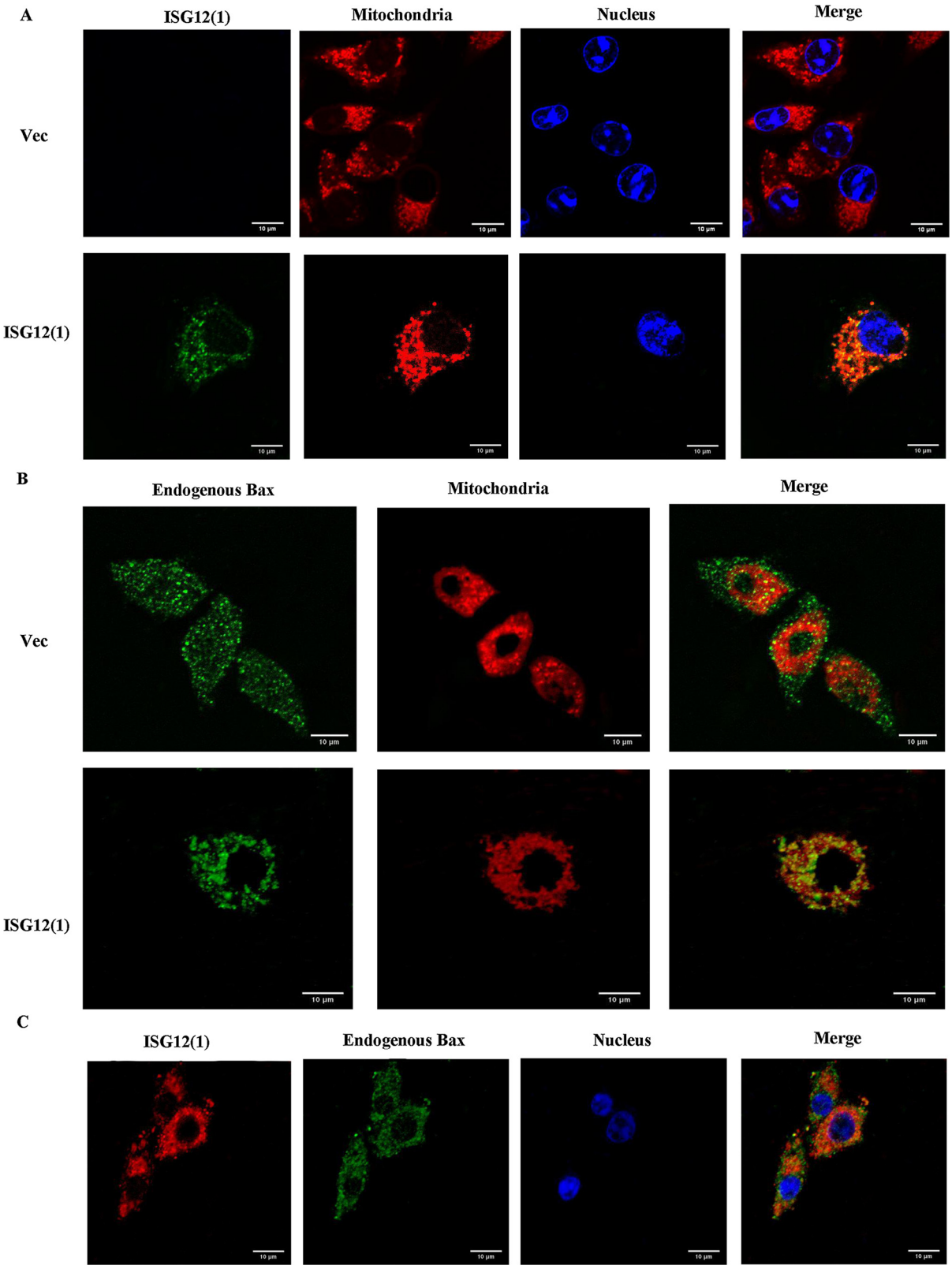

Fig. 4. Subcellular location of ISG12(1). (A) DF-1 cells were transfected with pCA-ISG12(1)-Flag or pCAGGS (Vec) and then stained with Mito-Tracker (red) and anti-Flag (green) antibodies, as well as Hoechst 33342. (B) DF-1 cells were transfected with pCA-ISG12(1)-Flag or pCAGGS (Vec) and then stained with Mito-Tracker (red) and anti-Bax (green) antibodies. (C) ISG12(1)-overexpressing cells were immunostained using anti-Flag (red) and anti-Bax (green) antibodies to assess the co-localization of both proteins. Then, fluorescence was detected by confocal microscopy. Scale bar: 10 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

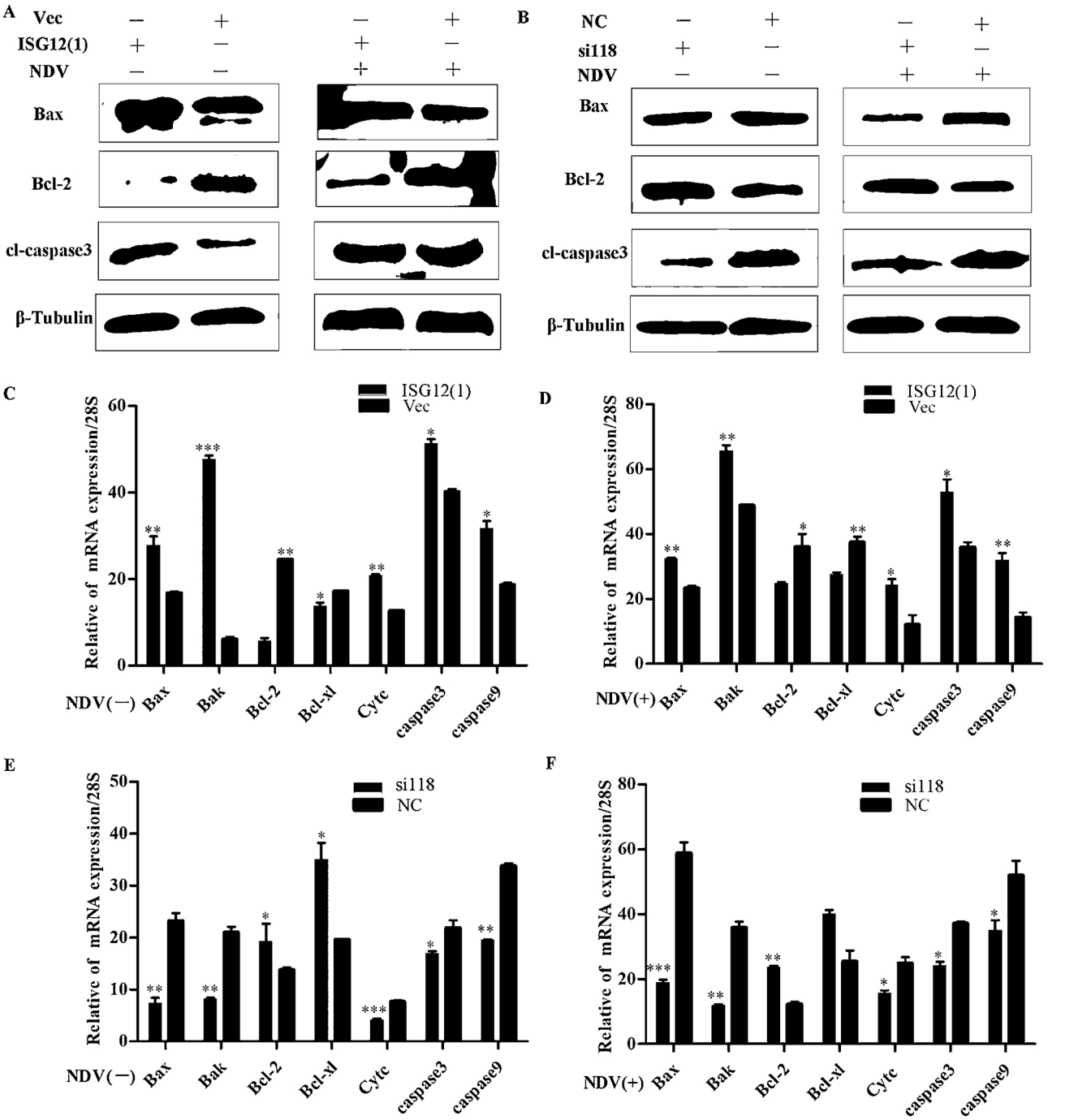

Fig. 5. ISG12(1) enhances Newcastle disease virus (NDV)-induced DF-1 cell apoptosis via the intrinsic apoptosis pathway.
Immunoblotting was used to detect the protein expression levels of Bax, Bcl-2, and cleaved caspase-3 in DF-1 cells transfected with pCA-ISG12(1)-Flag or ISG12(1) siRNA (si118) following infection with NDV (MOI = 0.1) or control treatment for 24 h (A and B). RT-qPCR was performed to analyze the mRNA expression levels of genes encoding Bax, Bak, Bcl-2, Bcl-xl, Cyt c, caspase-3, and caspase-9 in DF-1 cells overexpressing ISG12(1) with (C) or without (D) NDV infection or transfected with ISG12(1) siRNA (si118) with (E) or without NDV (F) infection 24 h after infection. Values are means ± SDs of three independent experiments.

activation of chicken ISG12(1) is not well discribed.

Using overexpression and knockdown approaches, we demonstrated that chicken ISG12(1) was involved in reducing NDV infection in DF-1 cells, which is consistent with a previous study, in which over-expression human ISG12a inhibited NDV replication in Huh7 and HLCZ01 cells(Liu et al., 2014a).ISG12 expression was also dramatically induced upon the infection with other viruses, such as DENV and HCV (Bièche et al., 2005; Lu et al., 2011). A recent study demonstrated that human ISG12a inhibits HCV replication through activation JAK-STAT signaling pathway (Chen et al., 2017). ISG12a was also reported to exert a direct antiviral effect by targeting HCV NS5 A for degradation (Xue et al., 2016). Here, we found that exogenetic expression chicken ISG12(1) promotes DF-1 cell apoptosis. In contrast, knockdown ISG12(1) expression reduced cell apoptosis, even after NDV infection. ISG12 regulation viruses replication via inducing cell apoptosis has been demonstrated previously. For example, miR-942 mediates HCV-induced cell apoptosis via targeting ISG12a (Liu et al., 2014b; Yang et al., 2014). In addition, mouse ISG12b2 involved in cells apoptosis process during DENV infection (Lu et al., 2011).

Cell apoptosis is a form of cell death regulated by many genes

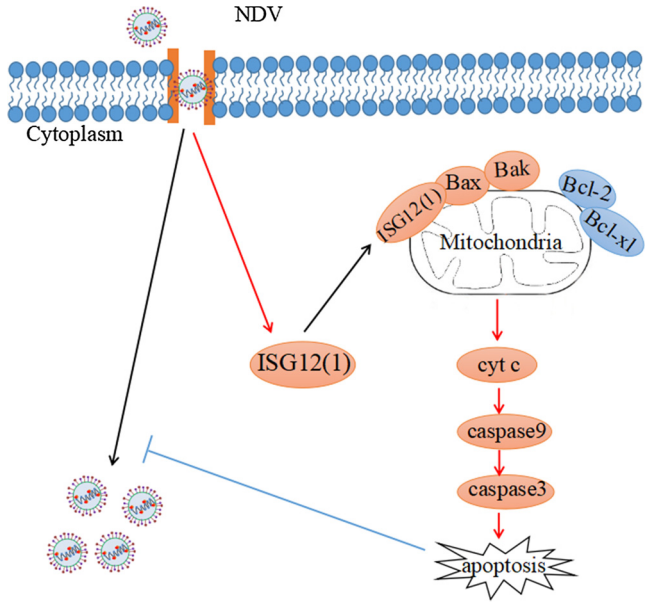

Fig. 6. Schematic model of ISG12(1) signal transduction pathway elicited in DF-1 cells by NDV.

(Elmore, 2007) that serves as a mechanism for the host to resist viral infection (Molouki et al., 2010). Cell apoptosis signal transduction can be roughly divided into two basic pathways: 1) the extrinsic FADD/caspase-8 signaling pathway, which is mediated by death receptors (Balachandran et al., 2000), and 2) the intrinsic caspase-9 pathway, which is regulated by increased mitochondrial outer membrane permeability (Panaretakis et al., 2003). In this study, we showed that chicken ISG12(1) localized to the mitochondria, suggesting that it might induce apoptosis in a mitochondria-dependent manner. When apoptosis occurs via the mitochondrial pathway, Bax and Bak form microporous channels in the mitochondrial outer membrane to increase membrane permeability, resulting in the release of protein molecules, such as Cyt c from the area between the mitochondrial inner and outer membranes to the cytoplasm. These proteins subsequently activate caspase-9 and caspase-3, resulting in the loss of normal morphology and eventually apoptosis (Tait and Green, 2013). We showed that ISG12(1) facilitates the translocation of Bax from the cytoplasm to mitochondria. Furthermore, overexpression of ISG12(1) activates the pro-apoptotic proteins Bak, Cyt c, and caspase-9/3, but inhibits the expression of anti-apoptotic proteins Bcl-2 and Bcl-xl. Lu et al. (2011) has suggested that mouse ISG12b2 interacts with the pro-apoptotic protein Bax to mediate cell apoptosis via the mitochondrial pathway. Similarly, in our study, ISG12(1) co-localized with Bax in the mitochondria. In future studies, the interaction of chicken ISG12(1) with Bax to induce cell apoptosis in response to NDV infection should be verified.

In conclusion, we confirmed that NDV infection induces high level expression of ISG12(1), and so as to decrease viral replication in host cells. The inhibitory effect of this protein on NDV was also found to depend on the mitochondrial pathway mediated cell apoptosis. These results provide new insight regarding the molecular mechanisms involving in NDV–host interactions and may contribute to the generation of new vaccines or other agents based on chicken ISG12(1) to control ND.

Competing interest

The authors declare that they have no competing interests.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (No. 31672581 to XingLong Wang and 31572538 to Zengqi Yang).

References

Alexander, D.J., 2000. Newcastle disease and other avian paramyxoviruses. Rev. Sci. Technol. 19, 443–462.

Amarasinghe, G.K., Aréchiga Ceballos, N.G., Banyard, A.C., Basler, C.F., Bavari, S., Bennett, A.J., Blasdell, K.R., Briese, T., Bukreyev, A., Cai, Y., Calisher, C.H., Campos Lawson, C., Chandran, K., Chapman, C.A., Chiu, C.Y., Choi, K.S., Collins, P.L., Dietzgen, R.G., Dolja, V.V., Dolnik, O., Domier, L.L., Dürrwald, R., Dye, J.M., Easton, A.J., Ebihara, H., Echevarría, J.E., Fooks, A.R., Formenty, P.B.H., Fouchier, R.A.M., Freuling, C.M., Ghedin, E., Goldberg, T.L., Hewson, R., Horie, M., Hyndman, T.H., Jiāng, D., Kityo, R., Kobinger, G.P., Kondō, H., Koonin, E.V., Krupovic, M., Kurath, G., Lamb, R.A., Lee, B., Leroy, E.M., Maes, P., Maisner, A., Marston, D.A., Mor, S.K., Müller, T., Mühlberger, E., Ramírez, V.M.N., Netesov, S.V., Ng, T.F.F., Nowotny, N., Palacios, G., Patterson, J.L., Pawęska, J.T., Payne, S.L., Prieto, K., Rima, B.K., Rota, P., Rubbenstroth, D., Schwemmle, M., Siddell, S., Smither, S.J., Song, Q., Song, T., Stenglein, M.D., Stone, D.M., Takada, A., Tesh, R.B., Thomazelli, L.M., Tomonaga, K., Tordo, N., Towner, J.S., Vasilakis, N., Vázquez-Morón, S., Verdugo, C., Volchkov, V.E., Wahl, V., Walker, P.J., Wang, D., Wang, L.F., Wellehan, J.F.X., Wiley, M.R., Whitfield, A.E., Wolf, Y.I., Yè, G., Zhāng, Y.Z., Kuhn, J.H., 2018. Taxonomy of the order mononegavirales: update 2018. Arch. Virol. 163, 2283–2294.

Balachandran, S., Roberts, P.C., Kipperman, T., Bhalla, K.N., Compans, R.W., Archer, D.R., Barber, G.N., 2000. Alpha/beta interferons potentiate virus-induced apoptosis through activation of the fadd/caspase-8 death signaling pathway. J. Virol. 74, 1513–1523.

Bièche, I., Asselah, T., Laurendeau, I., Vidaud, D., Degot, C., Paradis, V., Bedossa, P., Valla, D.C., Marcellin, P., Vidaud, M., 2005. Molecular profiling of early stage liver fibrosis in patients with chronic hepatitis C virus infection. Virology 332, 130–144.

Borden, E.C., Sen, G.C., Uze, G., Silverman, R.H., Ransohoff, R.M., Foster, G.R., Stark, G.R., 2007. Interferons at age 50: past, current and future impact on biomedicine. Nat. Rev. Drug Discov. 6, 975–990.

Chen, Y., Jiao, B., Yao, M., Shi, X., Zheng, Z., Li, S., Chen, L., 2017. ISG12a inhibits HCV replication and potentiates the anti-HCV activity of IFN-α through activation of the JAK/STAT signaling pathway independent of autophagy and apoptosis. Virus Res. 227, 231–239.

Cheriyath, V., Leaman, D.W., 2011. Emerging roles of FAM14 family members (GIP3/ISG 6-16 and ISG12/IFI27) in innate immunity and cancer. J. Interferon Cytokine Res. 31, 173–181.

Cho, H., Proll, S.C., Szretter, K.J., Katze, M.G., Gale Jr, M., Diamond, M.S., 2013. Differential innate immune response programs in neuronal subtypes determine susceptibility to infection in the brain by positive-stranded RNA viruses. Nat. Med. 19 (4), 458–464.

Chu, Z., Wang, C., Tang, Q., Shi, X., Gao, X., Ma, J., Lu, K., Han, Q., Jia, Y., Wang, X., Adam, F.E.A., Liu, H., Xiao, S., Wang, X., Yang, Z., 2018. Newcastle disease virus V protein inhibits cell apoptosis and promotes viral replication by targeting CacyBP/SIP. Front. Cell. Infect. Microbiol. 8 (304).

de Veer, M.J., Holko, M., Frevel, M., Walker, E., Der, S., Paranjape, J.M., Silverman, R.H., Williams, B.R., 2001. Functional classification of interferon-stimulated genes identified using microarrays. J. Leukoc. Biol. 69, 912–920.

Deist, M.S., Gallardo, R.A., Bunn, D.A., Kelly, T.R., Dekkers, J.C.M., Zhou, H., Lamont, S.J., 2017. Novel mechanisms revealed in the trachea transcriptome of resistant and susceptible chicken lines following infection with Newcastle disease virus. Clin. Vaccine Immunol. 24 00027–17.

Dimitrov, K.M., Ramey, A.M., Qiu, X., Bahl, J., Afonso, C.L., 2016. Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). Infect. Genet. Evol. 39, 22–34.

Dimitrov, K.M., Afonso, C.L., Yu, Q., Miller, P.J., 2017. Newcastle disease vaccines-a solved problem or a continuous challenge? Vet. Microbiol. 206, 126–136.

Dortmans, J.C., Koch, G., Rottier, P.J., Peeters, B.P., 2011. Virulence of Newcastle disease virus: what is known so far? Vet. Res. 42 (122).

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516.

Ganar, K., Das, M., Sinha, S., Kumar, S., 2014. Newcastle disease virus: current status and our understanding. Virus Res. 184, 71–81.

Haller, O., Weber, F., 2009. The interferon response circuit in antiviral host defense. Verh K Acad Geneeskd Belg 71, 73–86.

Hu, Z., Hu, J., Hu, S., Song, Q., Ding, P., Zhu, J., Liu, X., Wang, X., Liu, X., 2015. High levels of virus replication and an intense inflammatory response contribute to the severe pathology in lymphoid tissues caused by Newcastle disease virus genotype VIId. Arch. Virol. 160, 639–648.

Itsui, Y., Sakamoto, N., Kurosaki, M., Kanazawa, N., Tanabe, Y., Koyama, T., Takeda, Y., Nakagawa, M., Kakinuma, S., Sekine, Y., Maekawa, S., Enomoto, N., Watanabe, M., 2006. Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis C virus replication. J. Viral Hepat. 13, 690–700.

Jia, Y.Q., Wang, X.L., Wang, X.W., Yan, C.Q., Lv, C.J., Li, X.Q., chu, Z.L., xiao, S., zhang, S.X., yang, Z.Q., 2018. Common microRNA-mRNA interactions in different newcastle disease virus-infected chicken embryonic visceral tissues. Int. J. Mol. Sci. 19.

Kaiser, A., Willer, T., Sid, H., Petersen, H., Baumgärtner, W., Steinberg, P., Rautenschlein,

S., 2016. Susceptibility of primary chicken intestinal epithelial cells for low pathogenic avian influenza virus and velogenic viscerotropic Newcastle disease virus. Virus Res. 225, 50–63.
Kang, Y., Feng, M., Zhao, X., Dai, X., Xiang, B., Gao, P., Li, Y., Li, Y., Ren, T., 2016. Newcastle disease virus infection in chicken embryonic fibroblasts but not duck embryonic fibroblasts is associated with elevated host innate immune response. Virol. J. 13 (41).
Krishnamurthy, S., Samal, S.K., 1998. Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. J. Gen. Virol. 79, 2419–2424.
Labrada, L., Liang, X.H., Zheng, W., Johnston, C., Levine, B., 2002. Age-dependent resistance to lethal alphavirus encephalitis in mice: analysis of gene expression in the central nervous system and identification of a novel interferon-inducible protective gene, mouse ISG12. J. Virol. 76, 11688–11703.
Liu, S.Y., Sanchez, D.J., Cheng, G., 2011. New developments in the induction and antiviral effectors of type I interferon. Curr. Opin. Immunol. 23, 57–64.
Liu, W.Q., Tian, M.X., Wang, Y.P., Zhao, Y., Zou, N.L., Zhao, F.F., Cao, S.J., Wen, X.T., Liu, P., Huang, Y., 2012. The different expression of immune-related cytokine genes in response to velogenic and lentogenic Newcastle disease viruses infection in chicken peripheral blood. Mol. Biol. Rep. 39, 3611–3618.
Liu, N., Long, Y., Liu, B., Yang, D., Li, C., Chen, T., Wang, X., Liu, C., Zhu, H., 2014a. ISG12a mediates cell response to Newcastle disease viral infection. Virology 462–463, 283–294.
Liu, N., Zuo, C., Wang, X., Chen, T., Yang, D., Wang, J., Zhu, H., 2014b. miR-942 decreases TRAIL-induced apoptosis through ISG12a downregulation and is regulated by AKT. Oncotarget 5, 4959–4971.
Liu, W., Qiu, X., Song, C., Sun, Y., Meng, C., Liao, Y., Tan, L., Ding, Z., Liu, X., Ding, C., 2018. Deep sequencing-based transcriptome profiling reveals avian interferon-stimulated genes and provides comprehensive insight into Newcastle disease virus-induced host responses. Viruses 10 (162).
Lu, M.Y., Liao, F., 2011. Interferon-stimulated gene ISG12b2 is localized to the inner mitochondrial membrane and mediates virus-induced cell death. Cell Death Differ. 18, 925–936.
MacMicking, J.D., 2012. Interferon-inducible effector mechanisms in cell-autonomous immunity. Nat. Rev. Immunol. 12, 367–382.
Meyer, K., Kwon, Y.C., Liu, S., Hagedorn, C.H., Ray, R.B., Ray, R., 2015. Interferon-α inducible protein 6 impairs EGFR activation by CD81 and inhibits hepatitis C virus infection. Sci. Rep. 5 (9012).
Molouki, A., Hsu, Y.T., Jahanshiri, F., Rosli, R., Yusoff, K., 2010. Newcastle disease virus infection promotes Bax redistribution to mitochondria and cell death in Hela cells. Intervirology 53, 87–94.
Panaretakis, T., Pokrovskaja, K., Shoshan, M.C., Grandér, D., 2003. Interferon-alpha-induced apoptosis in u266 cells is associated with activation of the proapoptotic Bcl-2 family members Bak and Bax. Oncogene 22, 4543–4556.
Parker, N., Porter, A.C., 2004. Identification of a novel gene family that includes the interferon-inducible human genes 6-16, and ISG12. BMC Genomics 5 (8).
Pestka, S., Krause, C.D., Walter, M.R., 2004. Interferons, interferon-like cytokines, and their receptors. Immunol. Rev. 202, 8–32.
Qi, Y., Li, Y., Zhang, Y., Zhang, L., Wang, Z., Zhang, X., Gui, L., Huang, J., 2015. IFI6 inhibits apoptosis via mitochondrial-dependent pathway in Dengue virus 2 infected vascular endothelial cells. PLoS One 10.
Randall, R.E., Goodbourn, S., 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J. Gen. Virol. 89, 1–47.
Rasmussen, U.B., Wolf, C., Mattei, M.G., Chenard, M.P., Bellocq, J.P., Chambon, P., Rio, M.C., Basset, P., 1993. Identification of a new interferon-alpha-inducible gene (p27) on human chromosome 14q32 and its expression in breast carcinoma. Cancer Res. 53, 4096–4101.
Rue, C.A., Susta, L., Cornax, I., Brown, C.C., Kapczynski, D.R., Suarez, D.L., King, D.J., Miller, P.J., Afonso, C.L., 2011. Virulent Newcastle disease virus elicits a strong innate immune response in chickens. J. Gen. Virol. 92, 931–939.
Schneider, W.M., Chevillotte, M.D., Rice, C.M., 2014. Interferon-stimulated genes: a complex web of host defenses. Annu. Rev. Immunol. 32, 513–545.
Tait, S.W., Green, D.R., 2013. Mitochondrial regulation of cell death. Cold Spring Harb. Perspect. Biol. 5, 64–74.
Takaoka, A., Yanai, H., 2006. Interferon signalling network in innate defence. Cell. Microbiol. 8, 907–922.
Takeuchi, O., Akira, S., 2009. Innate immunity to virus infection. Immunol. Rev. 227 (75).
Xiang, B., Zhu, W., Li, Y., Gao, P., Liang, J., Liu, D., Ding, C., Liao, M., Kang, Y., Ren, T., 2018. Immune responses of mature chicken bone-marrow-derived dendritic cells infected with Newcastle disease virus strains with differing pathogenicity. Arch. Virol. 163, 1407–1417.
Xue, B., Yang, D., Wang, J., Xu, Y., Wang, X., Qin, Y., Tian, R., Chen, S., Xie, Q., Liu, N., Zhu, H., 2016. ISG12a restricts hepatitis C virus infection through the ubiquitination-dependent degradation pathway. J. Virol. 90, 6832–6845.
Yang, D., Meng, X., Xue, B., Liu, N., Wang, X., Zhu, H., 2014. MiR-942 mediates hepatitis C virus-induced apoptosis via regulation of ISG12a. PLoS One 9.
Zhang, S., Sun, Y., Chen, H., Dai, Y., Zhan, Y., Yu, S., Qiu, X., Tan, L., Song, C., Ding, C., 2014. Activation of the PKR/eIF2α signaling cascade inhibits replication of Newcastle disease virus. Virol. J. 11 (62).
Zhu, J., Hu, S., Xu, H., Liu, J., Zhao, Z., Wang, X., Liu, X., 2016. Characterization of virulent Newcastle disease viruses from vaccinated chicken flocks in Eastern china. BMC Vet. Res. 12 (113).