Molecular cloning and transcriptional regulation of Indian peafowl (*Pavo cristatus*) IFN- α gene

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Abstract

Interferon- α (IFN- α) resists viral infections by triggering the transcription of a diverse range of antiviral IFN-stimulated genes (ISGs). However, information about the Indian peafowl (*Pavo cristatus*) IFN- α (PcIFN- α) has not been reported. In this study, a PcIFN- α gene was amplified, which encoded a protein of 193 amino acids with a 26-amino acid signal peptide sharing 72.16–95.70% identity with other avians in Aves. After expression in prokaryote, PcIFN- α was analyzed for its physicochemical property and antiviral activity. Intriguingly, compared with chicken IFN- α , an effective viral infection therapeutic agent, PcIFN- α showed superior anti-VSV, NDV, and AIV activities, which were then abrogated by rabbit anti-PcIFN- α antibodies in vitro. Moreover, PcIFN- α was shown to be highly sensitive to trypsin; however, it remained stable despite changes in pH and temperature. Additionally, PcIFN- α induced the transcriptional or translational levels of Mx1 and ISG12 genes time-dependently. Overall, the present study revealed that PcIFN- α is a potential novel effective therapeutic agent in antiviral defense responses in peafowl, improving understanding of its involvement in bird antiviral defense.

Keywords *Pavo cristatus* · Interferon- α · Molecular cloning · Characterization · Interferon-stimulated genes

Introduction

The Indian peafowl (*Pavo cristatus*), a rare ornamental animal species, is a group of small, relatively somber forest pheasants in tropical Asia (Madge 2002). Recently, they gained considerable interest due to the train and keratin homogeneity of their tail feathers and courtship display (Pabisch et al. 2010). However, this species has been plagued by many diseases, including the Newcastle disease virus (NDV), which causes major losses and devastation to the poultry industry covering 200 species of birds (Alexander 1996). Lee et al. (2004) and Dou and Yang (2007) have isolated NDV from peafowls in Korea and China, respectively. Munir et al. (2012) have investigated a disease outbreak involving high mortality of peafowls in Pakistan with successful isolation of NDV. Additionally, herpesvirus, pox viruses, avian mycobacteriosis,

and *Eimeria pavonina* have long affected peafowl (Al et al. 1979; Hauck and Hafez 2012; Seimon et al. 2012; Stepien-Pysniak et al. 2016). Moreover, in a clinical case of *Chlamydophila psittaci* infection, severe pneumonia, airsacculitis, and diarrhea occurred in peacocks with 60% morbidity and 10% mortality. Additionally, six farmers who were in close contact with these infected peacock suffered severe acute respiratory syndrome characterized by pneumonia, high fever, and respiratory distress (Yang et al. 2011). Therefore, it is urgent to develop non-toxic and effective antiviral drugs to prevent and control infections in Indian peafowl.

Interferon (IFN), one of the first lines of host defense against invading pathogens, evolves to adapt with ever-changing viral threats during vertebrate speciation (Sang et al. 2014). Recently, interferon has been administrated as an immunomodulatory therapy (Domenech et al. 2011) to treat several immune-mediated and viral diseases by triggering the transcription of a diverse subset of antiviral IFN-stimulated genes (ISGs). Different types of IFNs exert their multifunctional activities by different induction of ISGs, establishing a cellular antiviral state. ISG-encoded proteins restrain pathogens by the inhibition of viral formation and the degradation of viral nucleic acids (MacMicking 2012). So far,

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human recombinant IFN- α s have been elaborated as part of the innate immune response which suppress influenza A virus replication and reduce disease in ferrets, mice, and non-human primates (Tumpey et al. 2007; Kugel et al. 2009; Matzinger et al. 2011). Also, porcine IFN- α controls viral diseases in pigs by stimulating the immune response, inhibiting replication of classical swine fever virus (Xia et al. 2005), foot and mouth disease virus (Dias et al. 2011), and porcine parvovirus (Liu et al. 2011).

IFN exerts natural immunity in disease resistance (Krieger and Ferreira 2003). Chickens, like peafowl, belong to the same order Galliformes. IFN in chicken (ChIFN) was discovered 60 years ago and was found to interfere with influenza virus replication in chick chorioallantoic membrane (Isaacs and Lindenmann 1957). Abundant evidence has demonstrated that ChIFN- α inhibits the replication of many epidemic avian viruses, including infectious bursal disease virus (IBDV), avian influenza viruses (AIV), Marek's disease virus (MDV), infectious bronchitis virus (IBV), and NDV (Qu et al. 2013). However, there has not been much progress in the expression and characteristic analysis of *P. cristatus* IFN- α (PcIFN- α). Confronted by the scarce understanding of the IFN- α gene in the Indian peafowl and the part it might play in antiviral responses, in this study, we cloned and assessed its molecular phylogenetic characteristics for the first time. Then, we characterized the response patterns of Mx1 and ISG12 after PcIFN- α exposure to chicken embryo fibroblasts (CEFs). The current findings facilitate research on the type I IFN family and provide a reference for bird species protection.

Materials and methods

Cell and viruses

The erythrocyte of Indian peafowl was a gift from the Livestock Research Institute of the Heilongjiang Academy of Agricultural Sciences. Sampling times ranged from 6:00 to 12:45 central standard time in April 2013. Indian peafowls (female, 20–36 months old) were captured via a mist net and placed in cloth bags until venipuncture could be performed. About 1 ml of blood was obtained from individual bird via the wing vein and carefully transferred to tubes containing 2 mg EDTA. Vesicular stomatitis virus (VSV), NDV, AIV, specific pathogen-free (SPF) eggs, and two female rabbits (New Zealand White strain, 4 weeks old) were obtained from Harbin Veterinary Research Institute, CAAS. Chicken *Gallus gallus* IFN- α (ChIFN- α) was produced in our laboratory. This research was supervised by the Animal Care, Use and Ethics Committee of NEFU (approval no. UT-31; 20 June 2014).

Cloning and analysis of the gene sequences encoding PcIFN- α

Primers for PcIFN- α (Table 1) were designed referring to the ChIFN- α sequence in GenBank (GU119896). Using Easypure Blood Genomic DNA Kit (TransGen, Beijing), we extracted genomic DNA from the erythrocyte of Indian peafowls. The PCR product was subcloned into a cloning vector (pMD19-T, Takara, Japan) and then sequenced (BOSHI, China). Characteristics, including sequence analysis, prediction of signal peptide, analysis of glycosylation sites, construction of multiple alignments, and prediction of three-dimensional (3D) structure elements, were analyzed by bioinformatics software as previously described (Zhao et al. 2017). MrBayes v3.1 was used to perform phylogenetic analysis (Ronquist and Huelsenbeck 2003) based on Bayesian inferences (BI), and maximum likelihood (ML) was approached using PhyML3.1 (Guindon et al. 2010). BEAST v1.7 software was used to infer a time-measured phylogeny (Drummond and Rambaut 2007). The BI and BEAST calculations were run over two million generations, after which 25% of the trees were discarded as burn-in. In total, 19 interferon sequences were run over two million generations in BI and BEAST calculations.

Prokaryotic expression of PcIFN- α

The vector pET32a (Novagen, Darmstadt, Germany) was used to generate prokaryotic expression for PcIFN- α , which included an N-terminal detection and purification epitope (His-Tag). After being cut with EcoR I and Hind III, PCR products were then subcloned into pET32a vector and transformed into *Escherichia coli*/BL21. The recombinant PcIFN- α protein was expressed and purified as previously described (Tian et al. 2014). Finally, we obtained the soluble homogeneous protein with biological activities against PBS solution and stored at -80°C . Both PcIFN- α and ChIFN- α were diluted with PBS at the same concentration.

Production of rabbit anti-PcIFN- α antibodies

The standard antiserum preparation procedure is referenced in Tian et al. (2014). Two rabbits were immunized subcutaneously with 0.1 mg of the purified PcIFN- α protein emulsified with 50% Freund's complete adjuvant (Sigma, USA). After the first vaccination, the rabbits were boosted twice every 14 days. Ten days after the last injection, blood was collected from the rabbits' ear vessels. Antiserum against PcIFN- α was separated at 1000 g and 4°C for 10 min and frozen at 20°C .

Table 1 Primers used in PcIFN- α and mature peptide (mPcIFN- α) gene PCR assays

Experiment	Primer name	Primer sequence (5'-3')
Peptide clone	PcIFN- α -A	ATGGCTGTGCCTGCAAG
	PcIFN- α -B	CTAAGTGC GCGTGTBB CB
Mature peptide clone	mPcIFN- α -A	<u>GAATTCT</u> GCAACCACCTTCGCCCCAG
	mPcIFN- α -B	<u>AAGCTT</u> CTAAGTGC GCGTGTGCCTGTGAG
qPCR of ISGs	PcMx-1-A	CTTTGTCTGGTGTTCCTCTTC
	PcMx-1-B	GCTGTATTTCTGTGTGCGG
	PcISG12-A	CAGTCTATCTCAATGGGTGGC
	PcISG12-B	CAGGTACAGGGAGAGCAAAG
	PcGAPDH-A	AGAACATCATCCCAGCGT
	PcGAPDH-B	AGCCTTCACTACCCTCTTG

Forward primer (mPcIFN-A) containing an EcoR I site (underlined), and reverse primer (mPcIFN-B) containing a Hind III site (underlined)

Western blotting analysis of PcIFN- α

Conditions and procedures of Western blotting were performed as in our previous study (Wang et al. 2018a). Six poly-histidine monoclonal antibodies (Zen BioScience, China) (1:500) were used as the primary antibodies.

Functional analysis of PcIFN- α

Endpoint dilution assay was used to determine tissue culture infectious dose 50 (TCID₅₀) of VSV, NDV, and AIV. The antiviral activities of PcIFN- α and ChIFN- α were determined and compared by cytopathic effect inhibition assays on CEFs. CEFs were freshly prepared from 9 to 11-day-old-embryonated SPF eggs. The cells were grown in Dulbecco's minimal essential medium (DMEM) (HyClone, Utah, USA) supplemented with 10% fetal bovine serum (HyClone, Utah, USA), 0.22% NaHCO₃, and 100 U/ml penicillin and streptomycin at 37 °C in a humidified 5% CO₂ incubator. Briefly, monolayer CEFs were seeded in a 96-well plate (100 μ l/well) for 24 h. Then, supernatant was replaced by 100 μ l serial dilutions of PcIFN- α or ChIFN- α (0.5 mg/ml). After 24 h incubation, an equal volume of fresh medium containing VSV, NDV, and AIV (100 TCID₅₀/well) was added. Simultaneously, positive (virus) and negative (IFN- α) controls were set. After observing the results with an inverted microscope for 12 to 72 h, the dilution of interferon to inhibit 50% of the cell lesion was defined as a unit of interferon.

An MTT assay was then used to determine cell viability. The CEFs were incubated with PcIFN- α (0.05 mg/ml) in a complete medium for 24 h, followed by incubation with 100 TCID₅₀ of VSV, AIV, and NDV for another 24 h, and then, the MTT solution (0.5 mg/ml) was treated for 4 h. The supernatants were removed carefully, followed by the addition of 100 μ l DMSO. The absorbance was measured at 570 nm and, then, calculated (Ruize, Tianjin, China).

To determine whether these antiviral activities were specific or not, an antibody blocking assay was measured as previously described (Shao et al. 2015) with some modifications. CEFs were treated with PcIFN- α (100 μ l) neutralized by rabbit anti-PcIFN- α antibody (1:20) for 4 h. Then, cells were challenged with 100TCID₅₀ of VSV. The negative control (preimmune rabbit serum) was set.

Primary physicochemical characteristics of PcIFN- α

Specifically, the PcIFN- α physicochemical characteristics, including the tolerance to trypsin, acidity and alkalinity, and temperature, were assessed using the CEFs/VSV system as described by Zhao et al. (2017).

PcIFN- α transcriptionally regulates the transcription of Mx1 and ISG12

Cultured CEFs were treated with PcIFN- α for 0, 3, 6, 9, 12, and 24 h. Subsequently, total RNA was extracted by RNAiso Plus reagent (Takara, Japan) and then reverse-transcribed by a PrimeScript RT Reagent Kit (Takara, Japan). QRT-PCR was carried out on a LightCycler® 480 (Roche, Switzerland) and determined with the FastStart Universal SYBR Green Master reagents (Roche, Switzerland). The PCR was set up under the following thermal cycling conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s (Wang et al. 2018b). Primers were as follows: PcGAPDH-A and PcGAPDH-B, PcMx-1-A and PcMx-1-B, and PcISG12-A and PcISG12-B (Table 1); these were then analyzed with the method $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen 2001).

PcIFN- α induced the protein expression of Mx1

CEF cultures as in previously were lysed in radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Beijing,

China). Lysates were collected and, then, separated by SDS-PAGE; PAb against Mx-1 (1:5000) (Proteintech, Wuhan, China) and GAPDH (1:8000) were used as the primary antibodies (Wang et al. 2018c).

Statistical analyses

Statistical analyses of all data were performed using SPSS (Inc., Chicago, IL, USA). All values were mean $\pm$ S.D. and assessed with ANOVA.

Results

Characterization of PcIFN- α gene

PcIFN- α was a 582-bp DNA sequence encoding 31 amino acids (AAs) for signal peptide and 162 for mature peptide (Fig. 1). The open-reading frame (ORF) of the PcIFN- α sequence has been uploaded to GenBank (KJ001188.1).

Three-dimensional structure prediction of PcIFN- α revealed that five alpha helices were consistent with the IFN

molecules analyzed by nuclear magnetic resonance spectroscopy (Ghasriani et al. 2013) (Fig. 1c), such as bovine IFN- ϵ (Guo et al. 2015), Amur tigers IFN- α (Zhao et al. 2017), and Whooper swan IFN- α (Zhao et al. 2018). Moreover, the investigation of secondary structure also indicated that PcIFN- α has five putative alpha helices which were labeled as A to E (Fig. 1b). Like most secreted proteins in eukaryotes, PcIFN- α contained a glycosylation site located in ⁷¹NYT⁷³ (Fig. 1b) as well as six cysteine residues located in 32, 61, 68, 100, 129, 155, and 168, indicating the existence of several disulfide bonds. In a nucleotide evolution tree analysis, a nearer relationship between PcIFN- α and other Aves IFN- α s than that of mammalian was displayed among a total of 19 interferon sequences (Fig. 2a). Specifically, PcIFN- α is clustered with interferon of chicken, quail, and *Chinese francolin*, which represents an in-group with respect to other Aves species. Specifically, the AA alignment showed that PcIFN- α identified 93.81% to ChIFN- α , 92.23% identical to *Chinese francolin* IFN- α , 87.88% identical to *Coturnix japonica* IFN- α , and 72.34% identical to *Goose* IFN- α (Fig. 2b).

Fig. 1 Cloning, sequence analysis, and 3D prediction of the PcIFN- α . **a** Amplified PCR product of mature. Lane M, Trans 2 K DNA marker; lane 1, complete sequence of PcIFN- α gene. **b** The sequence from 1 to 31 AA is the signal sequence. Cysteine residues forming disulfide bonds are marked with gray circles. Letters A–E refer to α -helices in PcIFN- α . **c** Predicted 3D structures of PcIFN- α . The five α -helices were labeled A–E

Fig. 2 Phylogenetic tree construction of the PcIFN- α and IFN- α AA alignment. **a** Phylogenetic tree based on combined Neighbour-Joining (NJ), Bayesian Inference (BI) and Maximum Likelihood (ML) methods using a GTR + G + I model for each tree. The NJ/ML bootstrap support is

indicated above the tree branch and BI posterior probability support underneath the branch. **b** IFN- α AA alignment of chicken, *Coturnix japonica*, *Chinese francolin* and goose. Identical residues are in black boxes, and similar residues are in grey

Fig. 3 Protein expression and verification of recombinant PcIFN- α . **a** Lane M: Unstained protein marker; Lane 1: Empty vector pET-33a (+); 2: rHis-PcIFN- α without isopropyl β -D-1-thiogalactopyranoside (IPTG) induction; 3: rHis-PcIFN- α after induction; 4: Sedimentations of rHis-

PcIFN- α ; 5: Supernatants of rHis-PcIFN- α ; **b** Lane 6: Purified rHis-PcIFN- α ; **c** Lane 7: Western blot analysis of His-tag recombinant PcIFN- α by 6-poly histidine monoclonal antibodies

Preparation of recombinant PcIFN- α

The mature peptide encoding PcIFN- α gene was cloned into the pET32a expression vector. Then, a recombinant protein PcIFN- α weighted apparent 38 kDa was mainly expressed and analyzed by SDS-PAGE (Fig. 3a, b). Western blotting using rabbit anti-PcIFN- α serum further corroborated that PcIFN- α gene was expressed and secreted (Fig. 3c).

Functional analysis of PcIFN- α

An endpoint dilution assay showed that TCID₅₀ of VSV, NDV, and AIV titers were 1.05×10^6 , 5.6×10^5 , and 7.2×10^6 U/0.1 ml, respectively. To determine whether PcIFN- α possesses biological function, we detected its antiviral activity and made a comparison with ChIFN- α using a cytopathic inhibition test. Renatured IFN- α s were diluted by four-fold dilution and preincubated in CEFs. After 24 h incubation of VSV, NDV, and AIV, the positive control displayed completely 100% cytopathic, and the dilution could confer a protective effect on CEFs to a certain extent. Negative controls were

normal in CEFs. Moreover, PcIFN- α exerted superior antiviral activity when CEFs were suffered by VSV, NDV, and AIV (1.09×10^4 , 2.84×10^4 , 6.45×10^4 U/mg, respectively) compared with ChIFN- α (9.94×10^3 , 4.75×10^3 , 4.64×10^3 U/mg, respectively) (Table 2). A neutralization assay in vitro demonstrated that PcIFN- α antiviral protective activities were aborted by rabbit anti-PcIFN- α antibodies (Fig. 4).

Cell viability was measured by the MTT assay. As shown in Fig. 5, exposure to VSV, NDV, and AIV (100 TCID₅₀) for 12 h resulted in a significant decrease of CEF viability to 79.8, 87.8, and 87.4% of normal. PcIFN- α at concentrations of 0.05 mg/ml significantly increased cell viability to 100.0, 115.6, and 118.1% of normal, respectively.

Primary physicochemical of PcIFN- α

Results showed that PcIFN- α was stable at changing pH (2–12) and temperature (42–65 °C) for 2 h ($P > 0.05$) (Fig. 6). On the contrary, the antiviral activity was completely aborted (data not shown) after treatment with 0.25% trypsin, confirming a common characteristic of interferons (Zhao et al. 2018).

PcIFN- α -induced the production of ISGs

The transcriptional level of ISGs (Mx-1 and ISG12) increased steadily from 0 to 24 h in PcIFN- α -treated CEFs (Fig. 7a). Western blotting displayed that the Mx-1 (76 kDa) protein showed the same trends from the 6 h after PcIFN- α treatment (Fig. 7b).

Table 2 Antiviral activity analysis for PcIFN- α and ChIFN- α

Measurement system	Antiviral activity (U/mg)	
	PcIFN- α	ChIFN- α
CEF-VSV	1.09×10^4	9.94×10^3
CEF-AIV	2.84×10^4	4.75×10^3
CEF-NDV	6.45×10^4	4.64×10^3

Fig. 4 Neutralization of PcIFN- α antiviral protective activity. **a** Negative control (PcIFN- α only); **b** PcIFN- α and VSV; **c** PcIFN- α and VSV preincubated with rabbit anti-PcIFN- α antibody; **d** positive control (VSV only) (TCID₅₀ of VSV titers was 1.048×10^6 U/0.1 ml)

Fig. 5 Effects of PcIFN- α against VSV-, NDV, and AIV-induced cytotoxicity in CEFs. Mean percentage of cell survival in the control group was scaled to 100%. C, control group (CEFs only); IFN, PcIFN- α (PcIFN- α only); Co, co-treatment group (virus and PcIFN- α)

Fig. 6 **a** pH and **b** temperature sensitivities of PcIFN- α . PcIFN- α was adjusted to pH 2.0, 4.0, 10.0, and 12.0 at 42, 53, and 65 °C and, then, finally reverted to the original pH (7.0) or cooled down in an ice box. Afterwards, antiviral activities were separately analyzed in F81/VSV system

Discussion

Dwelling in cultivated areas and stream banks, Indian peafowl is also known to forage for scraps around human habitats and villages. The movement of this species, no matter in the zoo or wild, is in close proximity to domestic birds, most of which are always threatened to the point of becoming a disease outbreak (Khulape et al. 2014). As an economically important virus affecting the poultry industry across the globe, the latest outbreak of NDV occurred during the summer months of 2012, and there was huge peacock mortality (Khulape et al. 2014). IFN, a cytokine responsible for resisting viral infections, has recently been re-investigated intensively. It became more evident as the interdependence of innate/adaptive immune responses (Gonzalez-Navajas et al. 2012). Interests in bird IFNs have recently sparked due to increasing viral diseases in the poultry industry. Recombinant ChIFN- α has been

found to exert anti-replication of various viruses in different cell lines (Qu et al. 2013). However, information about Indian peafowl IFN- α has not been available.

Although intronless and intron-containing type I IFN genes coexist in some amphibians, such as *Xenopus tropicalis* (Gan et al. 2017), generally, those in birds and mammals have the typical intron-lacking feature (Xu et al. 2013), which makes the method in which we cloned the PcIFN- α gene from total DNA feasible. Using Indian peafowl total DNA, we successfully amplified the predicted 582 bp IFN- α gene coding 193 AAs, which contains one potential N-glycosylation site and conserved six cysteine residues. Phylogenetic analysis revealed that IFN- α s in avian and mammalian were significantly subdivided into two monophyletic lineages, including carnivores and herbivores. The IFN- α s similarity of *P. cristatus*, *Francolinus pintadeanus*, *C. japonica*, and *G. gallus* brought their grouping in correspondence with the herbivorous monophyletic group. Indeed, the evolution of the IFN- α gene family in eutherian species has been shaped by both gene conversion and gene duplication (Hughes 1995), which is further supported by present features of PcIFN- α sequences. As expected from the close phylogenetic relationship, the highest homology was observed with the *G. gallus* and *F. pintadeanus* IFN- α sequences on the DNA as well as at the protein level (more than 90%), suggesting a common ancestor during evolution.

Recently, VSV has been reported to induce most of the cases of vesicular disease in livestock (Rodriguez 2002). In addition, type I IFN has been shown to have a key role in clearing VSV infection in mice (Muller et al. 1994). AIV is one of the major pathogens that significantly impacts the poultry industry worldwide (Olsen et al. 2006). In a recent in vitro assay determined in duck embryo fibroblasts, the anti-VSV and anti-AIV activities of rDuIFN- α were 2.1×10^5 and 4.1×10^5 U/mg, respectively (Gao et al. 2018). To explore the biological activities of PcIFN- α , the cytopathic inhibition insulted by VSV, AIV, and NDV was conducted

Fig. 7 Analysis of ISG transcription and Mx-1 protein expression in CEFs after PcIFN- α treatment. **a** Transcription analysis of ISGs in CEFs after PcIFN- α treatment. **b** Expression analysis of Mx-1 protein in CEFs after PcIFN- α treatment. CEFs without PcIFN- α treatment were used as control, while GAPDH was used as the internal reference. Data are presented in mean $\pm$ SD (* P < 0.05; ** P < 0.01)

and compared with another antiviral agent, ChIFN- α . Results showed that PcIFN- α exerted superior antiviral activity against VSV, AIV, and NDV (1.09×10^4 , 2.84×10^4 , and 6.45×10^4 U/mg, respectively) than did ChIFN- α (9.94×10^3 , 4.75×10^3 , 4.64×10^3 U/mg, respectively), indicating a certain degree of cross-species activity of PcIFN- α . These findings were logical considering the close relationship in evolutionary terms between peafowl and chicken (Table 2). An MTT assay also indicated that PcIFN- α could restore CEFs to normal activity when facing VSV, AIV, and NDV insults (Fig. 5). Similarly to ChIFN- α , PcIFN- α displayed low cytotoxicity in CEFs (Fig. 4a). In addition, PcIFN- α was found to be stable despite changes in pH and temperature while highly sensitive to trypsin. Given its substantial antiviral activity and low cytotoxicity, PcIFN- α becomes a potential candidate for antiviral therapeutic agent for peacocks and chickens.

As type I IFNs, IFN- α firstly binds with specific transmembrane receptors and exhibits antiviral activity by stimulating various downstream ISG expression (Schneider et al. 2014). At an early stage of viral infection in chicken, a number of ISGs, including the Mx1 and ISG12, were induced (Feng et al. 2017) to inhibit virus release or protein lysis, thus enhancing innate immune signaling and antiviral functions (Liu et al. 2015). As one of the first depicted inhibitors of virus invasion, the Mx1 gene product broadly inhibits genome replication at an early post-entry step of the virus life cycle (Schneider et al. 2014). ISG12 has also been shown to inhibit NDV replication (Liu et al. 2014). To depict the antiviral mechanism of PcIFN- α , these two ISGs were detected using qPCR or Western blot. Results showed that both Mx-1 and ISG12 were obviously induced from 6 h by PcIFN- α manifested by inhibiting replication of VSV, AIV, and NDV. Given that ISGs enhance innate immune signaling, PcIFN- α can further inhibit viral replication time-dependently by transcriptionally regulating this downstream antiviral gene expression. These observations mirror previous researches on Aves (whooper swan *Cygnus cygnus*) (Zhao et al. 2018) or mammalian (bovine) (Kim et al. 2013; Guo et al. 2016).

In conclusion, we analyzed the characterization and biological activity of PcIFN- α , which displayed strong antiviral activity against VSV, AIV, and NDV with low cytotoxicity and stable physicochemical characteristics. Additionally, PcIFN- α induced the transcriptional or translational levels of Mx-1 and ISG12 genes in a time-dependent manner. Overall, the present study on PcIFN- α enriched the current knowledge about the avian IFN- α family. All the consequences in our study provide baseline data for further research on PcIFN- α in the future, and these might provide clues to new preventative and therapeutic treatments for the poultry.

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