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Duck interferon regulatory factor 7 (IRF7) can control duck Tembusu virus (DTMUV) infection by triggering type I interferon production and its signal transduction pathway

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ABSTRACT

Human interferon regulatory factor 7 (IRF7) plays an important role in the innate antiviral immune response. To date, the characteristics and functions of waterfowl IRF7 have not been clarified. This study reports the cDNA sequence, tissue distribution, and antiviral function of duck IRF7. The duck IRF7 gene has a 1536-bp open read frame (ORF) and encodes a 511-amino acid polypeptide. IRF7 is highly expressed in the blood and pancreas of 5-day-old ducklings and in the small intestine, large intestine and liver of 60-day-old adult ducks. Indirect immunofluorescence assay (IFA) showed that over-expressed duck IRF7 was located in both the cytoplasm and nucleus of transfected duck embryo fibroblasts (DEFs), which was also observed in poly(I:C)-stimulated or duck Tembusu virus (DTMUV)-infected DEFs. Titres and copies of DTMUV were significantly reduced in DEFs over-expressing IRF7. Moreover, overexpression of duck IRF7 significantly induced IFNα/β, but not IFNγ, mRNA expression, and transcription of downstream interferon-stimulated genes (ISGs), such as MX, OASL and IL-6, which were significantly induced by poly(I:C) co-stimulation, was enhanced. Additionally, duck IRF7 over-expression can significantly activate the IFNβ promoter in DEFs. Collectively, duck IRF7 plays an important role in host anti-DTMUV immune regulation, which depends on type I interferons and associated signal transduction pathway(s).

1. Introduction

The innate immune response provides the first line of host defence during infection. Interferons (IFNs) are critical factors of the innate immune response [1,2], the initiation of which response depends on recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) [3]. Toll-like receptors (TLRs) and retinoic-acid-inducible gene I (RIG-I) are key PRRs in the host anti-dsRNA viral response [4,5] and can recruit downstream factors when activated, resulting in production of interferon and transcription of interferon-stimulated genes (ISGs) [2,6].

First cloned in 1997 [7], human IRF7 is the common downstream factor in both the TLR and RIG-I signalling pathways [8,9]. All IRF7s contain a conserved N-terminal DNA-binding domain (DBD) and an IRF-associated domain (IAD) [10], with IRF family members IRF7 and IRF3 having critical roles in type I IFN-mediated innate immunity. Recognition of pathogens by PRRs results in activation of IRF3 and/or IRF7 [11,12]. Phosphorylation activates IRF7, inducing dimerization and exposure of the nuclear localization signal (NLS) [2,8]. Activated IRF7/IRF3 then translocates to the nucleus and forms an enhanceosome with activating protein 1 (AP-1) and nuclear factor kappa B (NF-κB). These proteins bind to their respective positive regulatory domains, leading to recruitment of co-factors, resulting in transcription of type I IFNs [2].

First identified in 2010, duck Tembusu virus (DTMUV) is a newly emerging virus that leads to acute egg-drop syndrome in ducks [13]. DTMUV belongs to the *Flavivirus* genus and has a single positive-stranded RNA genome [14]. A recent report demonstrated that DTMUV can activate the host innate immune response through the MDA5 and TLR signalling pathways [15]. However, it remains unknown whether

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duck IRF7 is involved in those pathways.

Interestingly, IRF3 is absent in the avian genome [16], yet IRF7 may complement a portion of the IRF3 function [17]. Although chicken IRF7 has been identified [18], the characteristics and functions of IRF7 in waterfowl have not yet been elucidated. In this study, duck IRF7 was identified and cloned, and its tissue distribution in ducklings and ducks was examined. Duck IRF7 can activate the IFNβ promoter to induce expression of type I IFNs and ISGs. Duck IRF7 can also inhibit DTMUV replication in vitro, a process that plays an important role in viral infection.

2. Materials and methods

2.1. Experimental animals, virus, reagents

Healthy ducklings and ducks were purchased from a farm in Chengdu City, China. The duck Tembusu virus (DTMUV), CQW1 strain (NCBI accession No. KM233707.1) (50% *tissue culture infective dose* ($TCID_{50}$) = 2.5×10^6/mL), was provided by the Research Center of Avian Disease, Sichuan Agricultural University. Poly(I:C) was purchased from Sigma-Aldrich (USA).

2.2. Sequence amplification and plasmid construction

The reverse primer, IRF7-R (Table 1), for amplification of duck IRF7 was designed according to the predicted sequence of duck IRF7 (NCBI accession No. XM_021270589.1); the forward primer, IRF7-F (Table 1), was designed according the conserved domain between chicken and goose IRF7. The conditions used were 98 °C for 2 min, 98 °C for 10 s, 55 °C for 15 s, 72 °C for 20 s, and 72 °C for 5 min. The purified fragment was TA cloned into the pMD19-T simple vector (pMD™19-T Vector Cloning Kit, Takara, Japan). pMD19-T-IRF7 was used as the template and pCAGGS-IRF7-F and pCAGGS-IRF7-R as primers (Table 1) for polymerase chain reaction (PCR) amplification. Then, the fragment was cloned into pCAGGS vector with a Flag tag. IFN-β-Luc expresses firefly luciferase under the control of the duck IFN-β promoter (−96 to +1), which was constructed by our lab and described in a previous report [19]. pRL-TK was purchased from Promega (Wisconsin, USA).

2.3. Sequence analysis and phylogenetic analysis

The amino acid sequence of duck IRF7 was analysed using tools on the SMART website (http://smart.embl-heidelberg.de/). Amino acid sequence alignment including *Gallus gallus* (NP_990703.1), *Homo sapiens* (NP_001563.2) and *Mus musculus* (NP_058546.1) was conducted by Geneious software. A phylogenetic tree was constructed by the Neighbour-Joining method using Geneious software version 4.8.4 with the following alignment options: Blosum62 cost matrix (https://www.geneious.com/).

2.4. Tissue distribution profile of duck IRF7

Three 5-day-old ducklings and three 60-day-old adult ducks were sacrificed, and tissues (the small intestine, large intestine, muscle, bursa of Fabricius, thymus gland, liver, Harderian glands, spleen, pancreas, brain, trachea, glandular stomach, gizzard, kidney, heart, lung, blood, and lymph) were collected. Total RNA form the tissues was extracted using RNAiso Plus according to the manufacturer's instructions (Takara, Japan). For quantitative reverse transcription-PCR (qRT-PCR), 1 μg of total RNA was reverse transcribed following the standard protocol of PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Japan). The qRT-PCR reaction system included 0.4 μL of cDNA, 0.3 μL of qPCR-IRF7-F (Table 1), 0.3 μL of qPCR-IRF7-R (Table 1), 4 μL of RNAase-free ddH_2O and 5 μL of 2× EvaGreen qPCR Premix (Abcom, USA); the following cycling conditions were used: 95 °C for 10 min, 95 °C for 15 s, and 63 °C for 1 min.

2.5. Western blot analysis of duck IRF7 expression

Duck embryo fibroblasts (DEFs) were cultured in 6-well plates and transfected with 4 μg of pCAGGS-IRF7. After removing the culture medium, the cells were lysed with 500 μL of RIPA Lysis and Extraction Buffer (Thermo Fisher, USA). The cell lysates were separated by 12% polyacrylamide gel electrophoresis, and the separated proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Membranes were blocked overnight in 5% evaporated milk at 4 °C and incubated for 1 h with a 1:2000-diluted mouse anti-Flag (ProteinTech, China) or mouse anti-β-actin (Ruiying Biological, China) antibody at 37 °C. The membranes were then incubated for 1.5 h with 1:2000-diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Earthox, USA). The duck IRF7 protein was observed by enhanced chemiluminescence (Bio-Rad, USA), and images were acquired using ChemiDoc M (Bio-Rad, USA).

2.6. Cellular localization of duck IRF7 in DEFs

DEFs were cultured overnight in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS) and 5% CO_2 at 37 °C, and 2 μg/ml of plasmid of pCAGGS-IRF7 was transfected into cells at 70–90% confluence (Transintro EL transfection reagent, Transgene, China). At 24 h post-transfection, the DEFs were stimulated with DTMUV (25,000 TCID50) or 50 ng/mL poly(I:C) for 12 h, washed with phosphate-buffered saline/Tween 20 (PBST), fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton-X 100 in PBS, and blocked with 0.5% bovine serum albumin (BSA) for overnight. After three washes with PBS, the cells were incubated with mouse anti-Flag antibodies (Ruiying Biological, Suzhou, China) for 12 h at 4 °C. The cells were treated with goat anti-mouse Alexa 568 (Life Technologies) for 1 h and then with 4′,6-diamidino-2-phenylindole (Invitrogen) for 15 min at room temperature. These images were taken on a fluorescence microscope (Nikon, Tokyo, Japan).

2.7. Antiviral assay of duck IRF7 in vitro

DEFs at 70–90% confluence were transfected with pCAGGS-IRF7 or the pCAGGS empty vector as a negative control for 24 h, which were

Table 1
The sequence of Primers.

Primer	Sequence (5′ → 3′)
IRF7-F	GTGCGAGGGCAGCAGGGACA
IRF7-R	AGGGGCAGTGGGTGTCAGTC
pCAGGS-IRF7-F	CATCATTTTGGCAAAGAATTCGCCACCATGGCAGCGGCGGAGAGCGAAG
pCAGGS-IRF7-R	TTGGCAGAGGGAAAAAGATCTCTA CTTATCGTCGTCATCCTTGTAATCGTCTATCTGCATGTTGTACTGCTCGATGAGC
qPCR-IRF7-F	CGCCACCCGCCTGAAGAAGT
qPCR-IRF7-R	CTGCCCGAAGCAGAGGAAGAT
TMUV-E-qPCR-F	AATGGCTGTGGCTTGTTTGG
TMUV-E-qPCR-F	GGGCGTTATCACGAATCTA

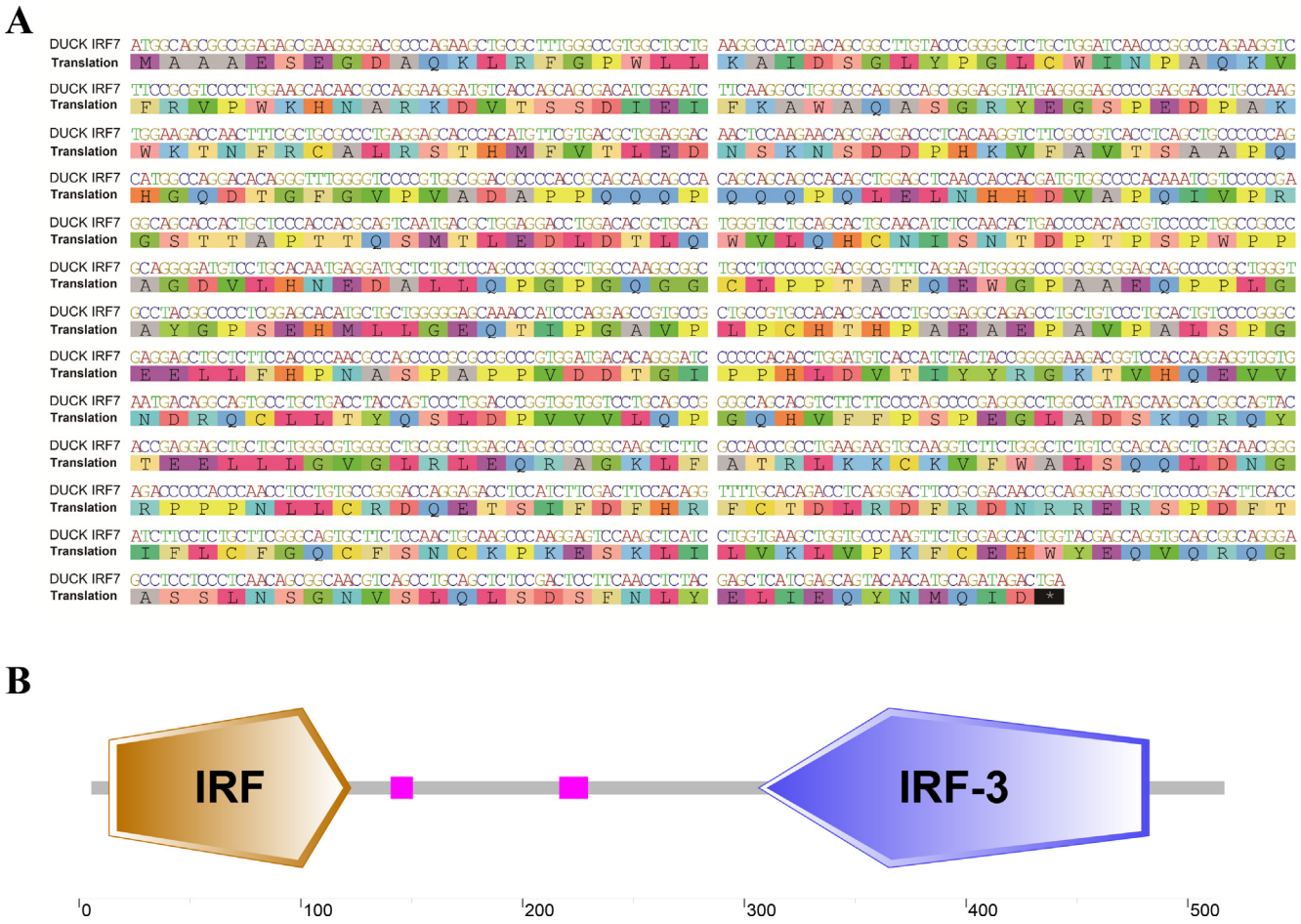

Fig. 1. Structural characterization of duck IRF7. (A) The duck IRF7 full-length cDNA and polypeptide. * is the termination codon. (B) Predicted functional domain map. Conserved functional domains were analysed using the amino acid sequence of duck IRF7 and the SMART website. Predicted functional domains are shown brown and blue. Low-complexity regions are shown in purple. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

infected with DTMUV (25,000 $TCID_{50}$). The infected cells were collected at different time points (36 h, 48 h), and virus titres were calculated by determining the $TCID_{50}$ using a previously described method [20]. An absolute quantitative curve was built based on the pCAGGS-DTMUV-E plasmid. The plasmid was diluted 10-fold; the temperature was auto-optimized, and the DTMUV standard curve was generated from 10^8 copies to 10^3 copies. Viral RNA from infected cells was extracted and reverse transcribed. The copy numbers were detected by qRT-PCR under the following conditions: 95 °C for 10 min, 95 °C for 15 s, and 63 °C for 1 min. The primers used are listed in Table 1.

2.8. The effects of duck IRF7 on subsequent cytokine mRNA expression

DEFs at 70–90% confluence were transfected with pCAGGS-IRF7 or the pCAGGS vector as a negative control for 24 h; poly(I:C) up to 50 μg/mL was added to some cells. At 24 h post-stimulation, the cells were collected, and total RNA was extracted. The mRNA expression levels of downstream factors were detected by qRT-PCR, as described above.

2.9. Luciferase reporter assays

DEFs were cultured in 48-well plates, and cells at 70–90% confluence were co-transfected with 0.4 μg/well of pCAGGS-IRF7 plasmid or empty control plasmid, together with the reporter plasmid IFN-β-Luc and pRL-TK plasmid according to the standard protocol. The pRL-TK plasmid was used as the internal control. The cells were harvested 48 h post-transfection, and firefly luciferase activity was measured according to the manufacturer's directions.

2.10. Statistical analysis

Student's *t*-test was used to evaluate differences. P-values < 0.05 were considered significant.

3. Results

3.1. Sequence analysis and phylogenetic tree

The cloned duck IRF7 cDNA is 1536 bp in length and encodes a 511-amino acid protein (Fig. 1A). As predicted by the SMART website, functional domains of duck IRF7 include the conserved IRF domain, which corresponds to the N-terminal DNA-binding domain (8–117 aa) present in all IRFs, and the IRF3 homologous domain at the C-terminus (301–477 aa) (Fig. 1B). Duck IRF7 shows 65.6% identity with chicken IRF7, 39.6% with human IRF7 and 36.7% with mouse IRF7 (Fig. 2A). Phylogenetic analysis of IRF7 grouped duck IRF7 and chicken IRF7 into the same clade (Fig. 2B).

3.2. Tissue distribution profile of duck IRF7

To investigate the levels of IRF7 mRNA in different tissues of ducklings or ducks, qRT-PCR was employed using duck β-actin as an

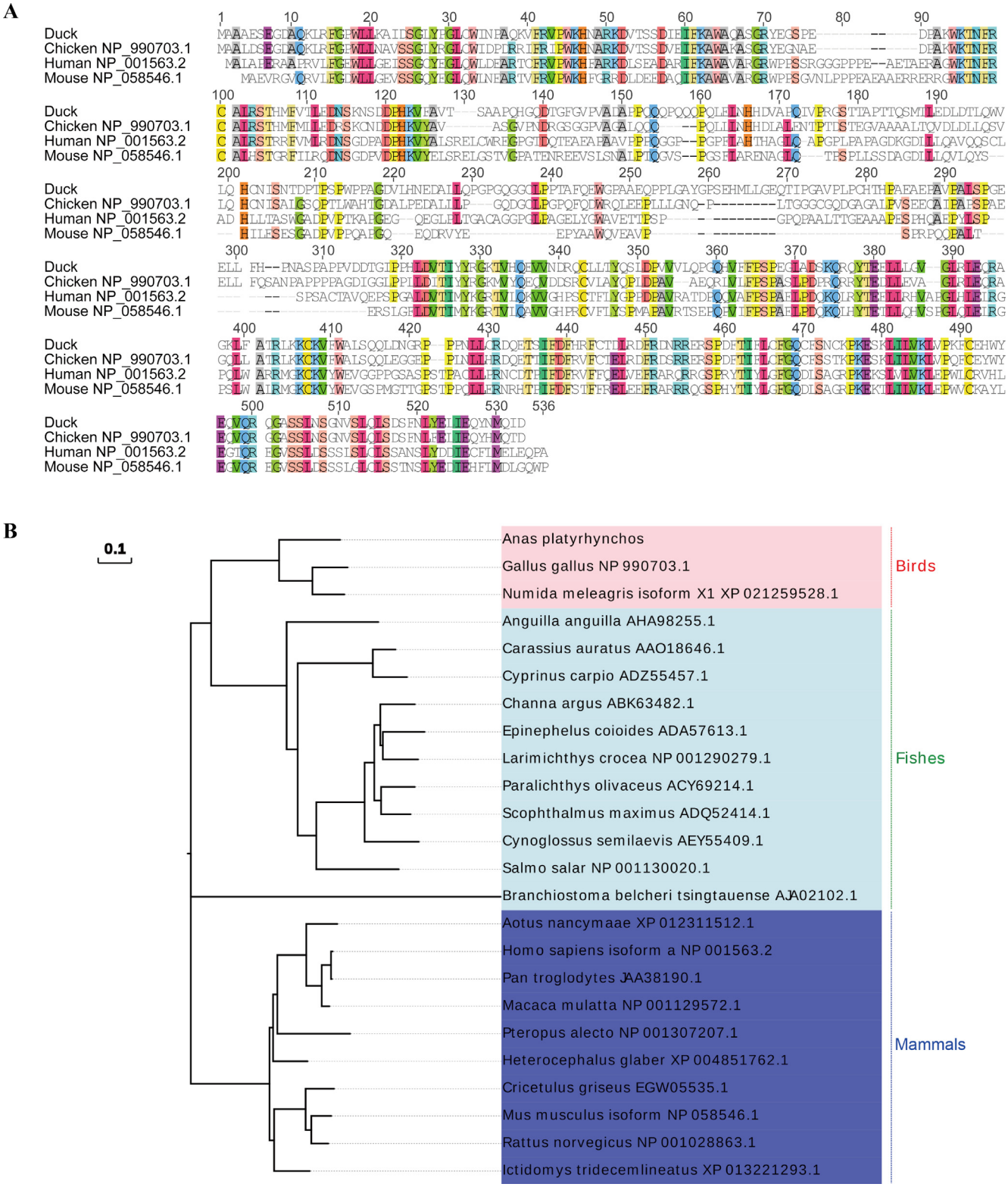

Fig. 2. Multiple sequence alignment and phylogenetic analysis of IRF7. (A) Polypeptide sequence of duck IRF7 was aligned with that of chicken (NP_990703.1), human (NP_001563.2) and mouse (NP_058546.1) using Geneious software. (B) The phylogenetic tree of IRF7, which was constructed by Neighbour-Joining using Geneious software version 4.8.4. The scale bar is 0.1.

internal control gene. In 5-day-old ducklings, the blood and pancreas showed high levels of expression (Red[2] Fig. 3); the kidney, lung, glandular stomach, Harderian gland, bursa of Fabricius, liver, trachea and heart had moderate transcription (Orange Fig. 3), whereas low levels were observed in the small intestine, brain, large intestine, muscle, spleen, thymus gland and gizzard (Purple Fig. 3). In 60-day-old ducks, high levels of transcription were found in the liver, small

[2] For interpretation of color in Fig. 3, the reader is referred to the web version of this article.

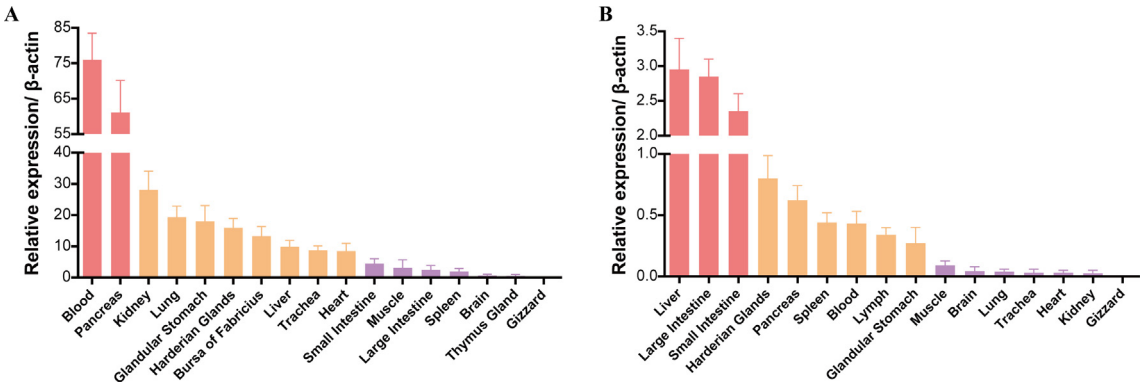

Fig. 3. Tissue distribution of duck IRF7. The relative expression levels of duck IRF7 in different tissues of 5-day-old ducklings (A) or 60-day-old ducks (B). qRT-PCR was performed to determine the transcription levels of IRF7 in tissues (the small intestine, large intestine, muscle, bursa of Fabricius, thymus gland, liver, Harderian gland, spleen, pancreas, brain, trachea, glandular stomach, gizzard, kidney, heart, lung, blood, and lymph) of ducklings or ducks; β-actin was chosen as the control gene. The data were analysed by GraphPad Prism software, and the results are presented as the mean ± SD (n = 3).

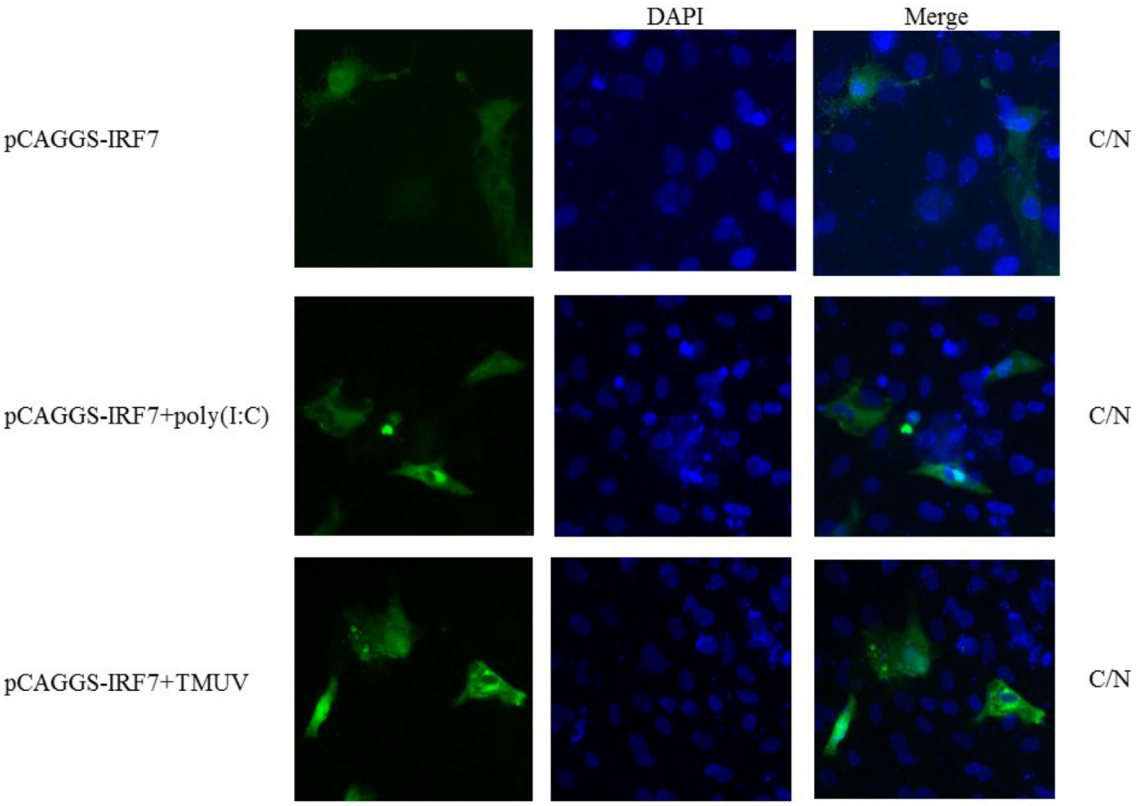

Fig. 4. Cellular localization of duck IRF7. pCAGGS-IRF7 was transfected into DEFs for 24 h, and the cells were then stimulated with DTMUV (25,000 $TCID_{50}$) or 50 ng/mL poly(I:C) for 12 h; cells were treated with blue 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence was detected by fluorescence microscopy. Magnification 600×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

intestine and large intestine (Red Fig. 3), with the Harderian gland, pancreas, spleen, blood, lymph and glandular stomach having moderate levels (Orange Fig. 3) and the muscle, brain, lung, trachea, heart, kidney and gizzard low levels (Purple Fig. 3).

3.3. Subcellular localization of duck IRF7

DEFs were cultured on cover slips in 12-well plates and transfected with 2 μg of pCAGGS-IRF7 plasmid for 24 h, after which the cellular location of IRF7 was determined by indirect immunofluorescence assay (IFA). IRF7 overexpression in DEFs resulted in two localization patterns: mainly distributed in the cytoplasm and well distributed in both the cytoplasm and nucleus (Fig. 4). DTMUV infection or poly(I:C) stimulation did not cause an obvious change in duck IRF7 overexpression in DEFs.

3.4. Antiviral function of duck IRF7

A significant decrease in DTMUV $TCID_{50}$ compared to the control was observed in cells overexpressing duck IRF7 at 36 h or 48 h post-infection (Fig. 5A). Furthermore, the copy numbers of DTMUV mRNA in the IRF7-overexpressing group were significantly lower than those in the negative control group (Fig. 5B). Expression of IRF7 at 24 h, 36 h and 48 h post-transfection was verified by western blotting (Fig. 5C).

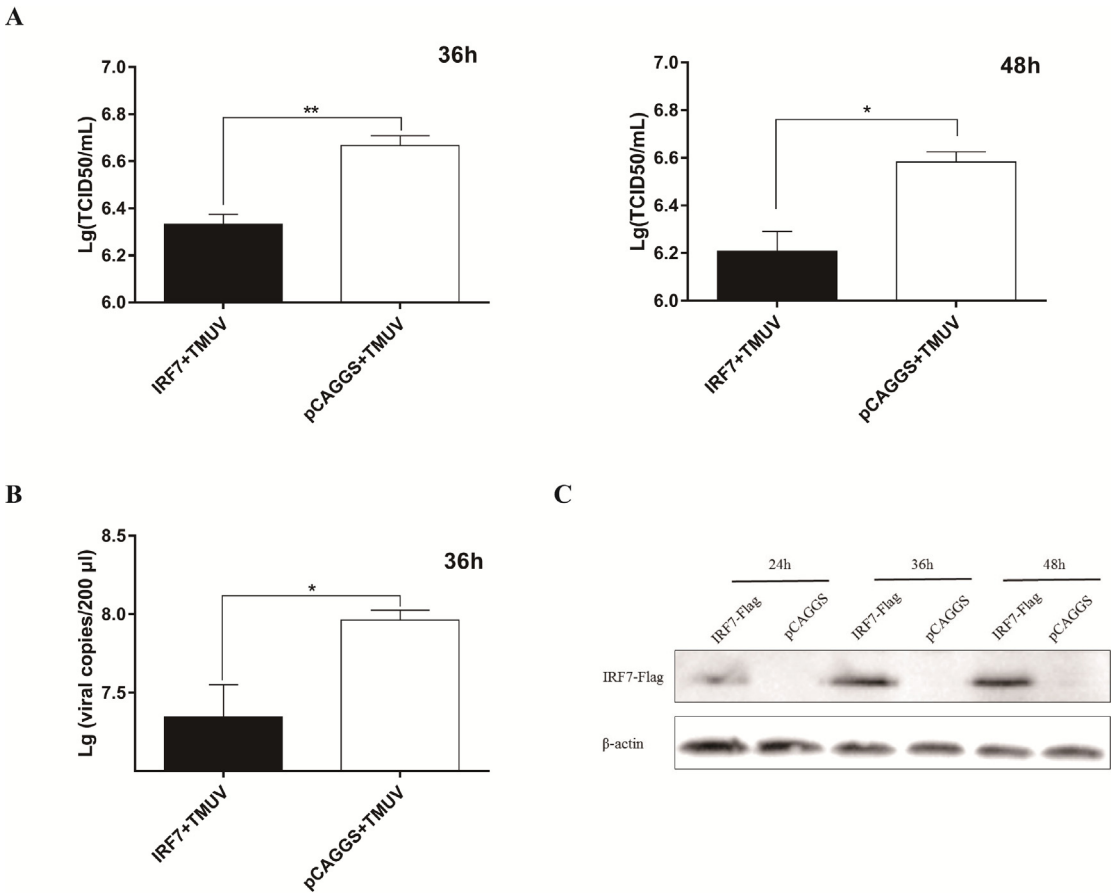

Fig. 5. The antiviral activity of duck IRF7 against duck DTMUV in vitro. (A) DEFs were cultured in 12-well plates and transfected with 2 μg of pCAGGS-IRF7 or the pCAGGS empty vector for 24 h and then infected with DTMUV (12,500 $TCID_{50}$). At 36 h and 48 h, the infected cells were collected, and the DTMUV $TCID_{50}$ was measured. (B) DEFs were cultured in 12-well plates, transfected with 2 μg of pCAGGS-IRF7 or the pCAGGS empty vector as the control for 24 h; the DEFs were then infected with DTMUV (12,500 $TCID_{50}$). At 36 h, the infected DEFs were harvested, and qRT-PCR was performed. (C) Duck IRF7 expression was confirmed by western blot analysis. DEFs were cultured in 6-well plates until 70–90% confluency and then transfected with 4 μg of pCAGGS-IRF7 or the pCAGGS empty vector as the control. The transfected DEFs were harvested at 24 h, 36 h and 48 h post-transfection; a mouse anti-Flag or anti-β-actin antibody was used as the primary antibody, and a goat anti-mouse antibody was used as the secondary antibody. The data were analysed by GraphPad Prism software, and are the results are presented as the mean ± SD (n = 3). ns: $P > 0.05$, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

3.5. IRF7 overexpression can trigger type I IFN expression and stimulate type I interferon and cytokine transcription

To verify whether IRF7 can regulate IFN and cytokine expression in duck cells, the mRNA levels of IFNs and cytokines were detected in IRF7-overexpressing DEFs with and without poly(I:C) stimulation. Type I interferons (IFNα and IFNβ) were significantly up-regulated in IRF7 + poly(I:C) groups, though there was no change in the level of IFNγ expression. The expression levels of MX, OASL, and IL-6 in IRF7 + poly(I:C) groups were significantly higher than those of either the pCAGGS + poly(I:C) group or IRF7 group; no difference between the poly(I:C) group and IRF7 overexpression group was observed (Fig. 6A).

To further verify that IRF7 can stimulate IFNβ transcription, DEFs were co-transfected with either pCAGGS-IRF7 or the pCAGGS empty vector and the reporter-IFNβ-promoter plasmid, and poly(I:C) was added at 24 h post-transfection. After 36 h, firefly luciferase and Renilla luciferase activities were measured. The relative light unit (RLU) of the duck IRF7 overexpression group was significantly higher than that of the control group (Fig. 6B). These results demonstrate that the up-regulation of IFN transcription was due to activation of the IFNβ promoter by duck IRF7 overexpression.

4. Conclusions

IRF7 has been identified in humans, fishes and birds. It is reported that chicken IRF7 can induce type I interferon transcription, thereby promoting expression of other ISGs in response to anti-Newcastle disease virus (NDV) in chicken embryo fibroblasts (CEFs) [21]. The antiviral function of IRF7 has also been verified in mice, humans and fishes [22–26]. Due to the lack of IRF3 in birds, IRF7 serves as a critical factor that can induce type I interferon transcription after activation by TBK1/IKKi or MAVS [27].

In this study, duck IRF7 was identified. The gene contains a 1536-bp ORF that encodes 511 amino acids. Using Geneious software for alignment, duck IRF7 exhibits 65.6% identity with chicken IRF7, 39.6% with human IRF7, and 36.7% with mouse IRF7. Phylogenetic analysis further showed that IRF7 groups closely with chicken and turkey IRF7. Multiple alignments of duck, chicken and mammalian IRF7 reveal the presence of a DNA-binding domain (DBD) at the N-terminus of duck IRF7, which contains a conserved Trp in all IRFs [28]. Based on the conserved DBD, IRFs can bind to nucleotide sequences such as IRF-binding elements, *interferon-sensitive response element* (ISREs) and positive regulatory elements [29]. A Ser-rich domain is also present at the C-terminus of duck IRF7, a region that is conserved in IRF3 and IRF7 and is the target of virus-induced phosphorylation [30]. Amino acid sequence analysis of duck IRF7 showed the same results, providing evidence for the function and activation mechanism of duck IRF7. High

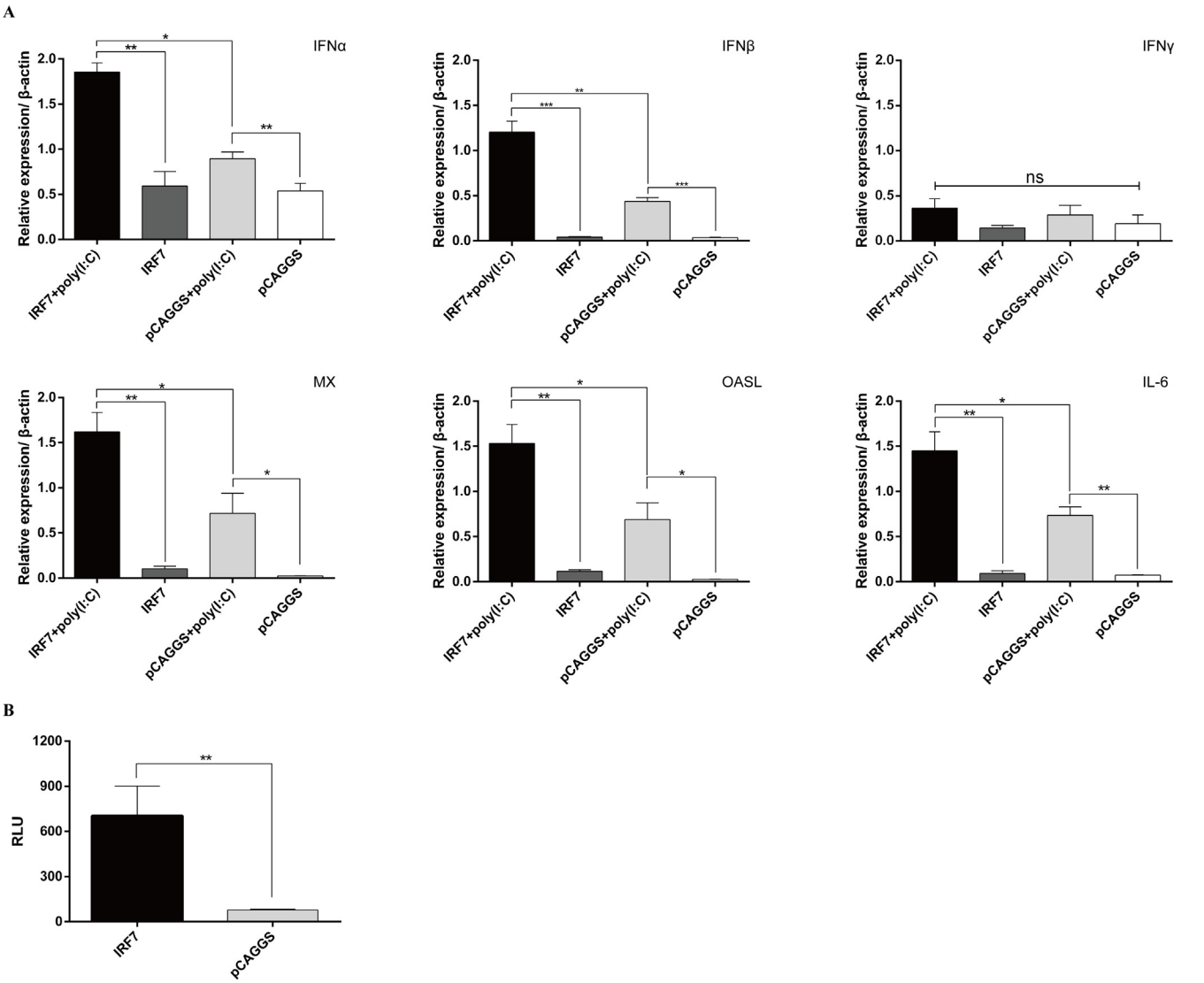

Fig. 6. (A) Relative expression of type I and II IFNs and downstream cytokines in IRF7-overexpressing DEFs. DEFs were cultured in 12-well plates, and pCAGGS-IRF7 or the pCAGGS empty vector was transfected. After 24 h, 50 μg of poly(I:C) was added, total RNA was extracted, and qRT-PCR was performed. (B) DEFs were cultured in 12-well plates until 70–90% confluency and co-transfected with 0.4 μg/well of the pCAGGS-IRF7 plasmid or empty control plasmid, together with the reporter plasmid IFN-β-Luc and pRL-TK plasmid. Firefly luciferase activities were measured at 48 h post-transfection. The data were analysed by GraphPad Prism software, and the results are presented as the mean ± SD (n = 3). ns: $P > 0.0$ *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

expression levels of IRF7 have been detected in the liver and heart in large yellow croaker [31] and the spleen in barbel chub [32]. In this study, IRF7 was found to be highly expressed in the blood and pancreas of 5-day-old ducklings, and in 60-day-old adult ducks, high levels were observed in the small intestine, large intestine and liver. In addition to roles in IFN regulation, IRF7 has multiple functions in mammals and is involved in regulating cell growth and apoptosis, susceptibility to and progression of cancer and immune cell differentiation and activation [29,33]. IRF7 expression was detected in most duck tissues, possibly demonstrating that duck IRF7 also has multiple functions.

Duck IRF7 with GFP at the C-terminus was used to determine the cellular localization of IRF7. The human IRF7-GFP fusion protein was observed in the cytoplasm of L929 cells [34], and chicken IRF7 was detected in the cytoplasm of CEFs [21]. As reported, IRF7 translocates to the nucleus when activated [21,30,34]. However, some reports indicate that non-activated IRF7 was detected in both the cytoplasm and nucleus. The rainbow trout IRF7-GFP fusion protein was observed mainly in the cytoplasm of RTG-2 cells, with weak nuclear expression observed [26]. In our study, overexpressed duck IRF7 was mainly detected in the cytoplasm but was also detected in the nucleus of DEFs, with little change after stimulation with DTMUV or poly(I:C).

The antiviral function of IRF7 has been reported in different animals, such as fishes, birds and mammals, by IRF7overexpression or knockdown [23–25]. DTMUV viral copy numbers in the duck were lower than those of the control group at 36 h after DTMUV infection. In addition, the IRF7-overexpressing group DTMUV $TCID_{50}$ was significantly lower than that of the control group at both 36 h and 48 h post-infection, indicating that duck IRF7 can inhibit DTMUV replication in DEFs.

To understand the molecular mechanism of duck IRF7 antiviral activity, DEFs were transfected with the pCAGGS-IRF7 plasmid, and poly(I:C) was added to stimulate cell signal transduction. Based on our data, duck IRF7 can up-regulate expression of type I IFN and ISGs, with no effects on type II IFN induction. Human IRF7 is activated by dimerization and phosphorylation [2,8]. As expected, in our study, type I IFNs and ISGs were only significantly up-regulated when duck IRF7-overexpressing cells were stimulated with poly(I:C), which may be because the function of duck IRF7 depends on the activities of upstream signalling factors.

In conclusion, this study reveals the tissue distribution of duck IRF7

mRNA expression, and the cellular localization of duck IRF7 was also determined. Based on our data, duck IRF7 can activate the IFNβ promoter to induce type I interferon (IFNα and IFNβ) transcription, inhibiting DTMUV replication in vitro. The findings provide useful information on the important role of duck IRF7 in protecting against avian flavivirus infection.

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