Distribution of Ifitm3 in Yellow-Feathered Broilers and Inhibition of Avian Reovirus Multiplication by Ifitm3

ABSTRACT

This study was carried to express the interferon-induced transmembrane protein 3 (IFITM3) in vitro and examine its function in inhibition of avian reovirus (ARV) replication. The recombinant prokaryotic vector expressing yellow-feathered broiler IFITM3 was successfully constructed, and the recombinant protein was expressed in competent Escherichia coli BL21 cells. New Zealand white rabbits were immunized with the purified recombinant protein to prepare a polyclonal antibody, with a titer of 1:128,000. Immunohistochemistry, reverse transcription–PCR, and real-time fluorescence quantitative PCR showed that IFITM3 was distributed in the yellow-feathered broiler immune organs, and the expression of IFITM3 in bursa of Fabricius was more than in spleen and thymus. It was found that in the thymus, spleen and bursa of Fabricius the mRNA expression levels of IFNα and IFITM3 were significantly induced after ARV infection. And it was also certified in the chicken embryo fibroblasts (CEFs) which infected with ARV. Then the IFNa was added into the cell culture medium before CEFs were infected with ARV. The results indicated that the mRNA of IFITM3 expression was significantly increased and ARV multiplication was significantly inhibited. And when the expression of IFITM3 was knocked down by siRNA-IFITM3, the expression of IFITM3 was significantly reduced, but the ARV multiplication was significantly increased, which indicated that IFITM3 protein could inhibit the ARV replication.

Abbreviations: avian reovirus, ARV; complementary deoxyribonucleic acid (cDNA); chicken embryonic fibroblasts, CEFs; double distilled water (ddH2O); Dulbecco’s modified Eagle medium, DMEM; glyceraldehyde-3-phosphate dehydrogenase gene, GAPDH; interferon alpha, IFNα; interferon-induced transmembrane protein, IFITM; intravenous, iv; median tissue culture infective dose, TCID50; multiplicity of infection, MOI; National Center of Biotechnology Information, NCBI; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; ribonucleic acid, RNA; reverse transcription, RT; sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE; small interfering RNAs (siRNAs).

INTRODUCTION

The innate immune system can recognize pathogen infections and then initiates the mechanisms that inhibit pathogen replication (Amanda et al., 2013). To defend against viral infection, the host will mobilize factors that challenge the virus. Interferons (IFNs) orchestrate a large component of the antiviral response (Brass et al., 2009), triggering
antiviral pathways by inducing the expression of interferon-stimulated genes (ISGs) (Randall et al., 2008). The interferon-inducible transmembrane (IFITM) proteins were first identified in IFN-treated neuroblastoma cells in 1984, and are encoded by a class of ISGs (Chen et al., 1984). The IFITM gene family includes IFITM1, IFITM2, IFITM3, IFITM5, IFITM6, IFITM7, and IFITM10 genes and some IFITM-like genes. These genes are induced by IFN and are widely expressed in tissues and organs, except IFITM5, which is only expressed in bone cells (Siegrist et al., 2011). The biological functions of IFITM3 include the inhibition of both viral replication and cell entry. Brass et al. (2009) demonstrated that the IFITM proteins inhibit the early replication of flaviviruses, including Dengue virus and West Nile virus. Lu et al. (2009) confirmed that IFITM proteins inhibit HIV-1 replication, at least partly by interfering with viral entry.

Infectious diseases greatly influence poultry production, especially viral infections that induce immune suppression. Avian reovirus (ARV) infections affect the poultry industry throughout the world (Lorena et al., 2009). ARV causes viral tenosynovitis, malabsorption, and respiratory diseases in chickens (Teng et al., 2013), and ARV-induced immunosuppression is one of the most important pathogenic mechanisms of the virus (Chen et al., 2015). However, how the innate immunity of chickens plays its role during ARV infection requires further study. Therefore, in this study, the IFITM3 gene of the yellow-feathered broiler was cloned, expressed, and confirmed, and the distribution of its expression in the chicken immune organs was investigated. And IFITM3 could inhibit ARV replication in vivo and in vitro that also was carried out.

**MATERIALS AND METHODS**

**Materials**

**Viruses**

Avian reovirus strain S1133 was propagated in chicken embryonic fibroblasts (CEFs), which were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, as previously described (Wang et al., 2012). The median tissue culture infective dose (TCID<sub>50</sub>) was 10<sup>-5.40</sup> (multiplicity of infection [MOI] = 8/mL).

**Cells**

CEFs were grown in Dulbecco’s modified Eagle medium (DMEM) containing streptomycin (50 μg/ml), penicillin G50 (50 μg/ml), and 5% foetal bovine serum, at 37 °C in 5% CO₂.

**Vectors**

The vectors used were the pGEM®-T Easy vector (Promega, Wisconsin, USA) and pET-32a(+) (Promega, Wisconsin, USA).

**Main reagents**

The main reagents used included the TIANamp® Viral Total Nucleic Acid Purification Kit (TIANamp, Beijing, China); the GoScript™ Reverse Transcription System (Promega, Wisconsin, USA); HilyMax™ Transfection Reagent (Dojindo, Shanghai, China); horseradish-peroxidase-labelled sheep anti-chick IgG antibody; Escherichia coli DH5α cells (Promega, Wisconsin, USA); E. coli BL21(Promega, Wisconsin, USA).

**Total RNA extraction and cDNA amplification**

Tissue samples (spleen, thymus, and bursa of Fabricius) were collected from healthy 20-day-old yellow-feathered broilers. Total RNA was extracted from the yellow-feathered broiler spleen with the TRIzol Plus RNA Purification Kit (TIANamp, Beijing, China). The first-strand cDNA was synthesized according to the manufacturer’s instructions. The total volume of the reverse transcription reaction mixture was 20 μl. The reaction was performed at 45 °C for 15 min and 72 °C for 15 min.

**Primers design**

Oligonucleotide primers for the PCR reactions and real time-PCR were designed (Table1). BstZ1 restriction sites were incorporated at the 5¢ ends of the IFITM3 gene primers.

**Cloning the chicken IFITM3 gene**

The PCR product amplified with the specific primers was purified and cloned into the pGEM®-T Easy vector (Promega, Wisconsin, USA). This recombinant plasmid was designated pGEM-T-IFITM3 and transformed into competent E. coli DH5α cells. Positive clones were selected with blue–white selection, and the positive plasmids were confirmed with PCR and sequencing.

The IFITM3 sequence was compared using the National Center for Biotechnology Information (NCBI) online BLAST tool. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were predicted with the CSS-1 software. The structure of the IFITM3 pro...
expression. The recombinant plasmid was designated PEASY™-E1–IFITM3 and was transformed into competent E. coli DH5α cells. Positive clones were selected with blue–white selection and confirmed with PCR (using the T7 upstream primer and the target gene downstream primer), sequencing, and restriction enzyme digestion. And PEASY™-E1–IFITM3 was transformed into Competent E. coli BL21(DE3) pLysS cells.

The pEASY™-E1–IFITM3-positive BL21(DE3) pLysS cells were cultivated for 14 h at 37 °C with 1 mmol/mL IPTG (Transgen Biotech, Beijing, China). The bacterial cells were collected and centrifuged at 12,000 g for 1 min. The pellet was then re-suspended in phosphate buffer (PBS) and broken with ultrasonography. The protein was denatured and identified with SDS-PAGE.

**Purification of recombinant IFITM3 (rIFITM3) and preparation of polyclonal antibody**

The recombinant protein was purified with affinity chromatography on ProteinInSo™ Ni-NTA Resin (Transgen Biotech, Beijing, China). rIFITM3 was identified with SDS-PAGE and Western blotting.

An anti-rIFITM3 polyclonal antibody was prepared as described previously (Zheng et al., 2014). Three New Zealand white rabbits were inoculated by subcutaneous injection with 1 mL (1 mg/mL) of purified IFITM protein, which was emulsified with an equal amount of Freund's complete adjuvant. Immunization was boosted twice by inoculation with 1 mL of antigen mixed with an equal volume Freund's incomplete adjuvant at 2-weekly intervals. The fourth immunization was performed 7 days after the third immunization, with 1 mL (1 mg/mL) of purified IFITM protein by intravenous (iv) injection.

The antibody titer was determined with the enzyme-linked immunosorbent assay.

**Immunohistochemical localization of chicken IFITM3 in immune organs**

The tissue samples including thymus, spleen, and bursa of Fabricius, were dehydrated in a graded series of ethanol, embedded in paraffin, and sectioned at 4–6 μm with a histotome. After dewaxing and hydration, the sections were analysed with immunohistochemistry method. For immunohistochemical analysis, the sections were incubated with the rabbit anti-IFITM3 antibody at 37 °C for 60-120 min. After rinsed with PBS for 5 min three times, the sections were incubated with a biotin-conjugated goat anti-rabbit antibody (Keygen Biotech Co. Ltd, Nanjing, China) at 37 °C for 10-30 min. After the sections were washed, they were treated with streptavidin-peroxidase for 10 min and then with a diaminobenzidine solution. The sections were then counterstained with haematoxylin and mounted with neutral gum.

**IFITM3 mRNA levels in chicken immune organs**

IFITM3 mRNA levels in chicken immune organs were examined by RT-PCR and SYBR Green real-time PCR. RT-PCR was performed with 1 μL of cDNA, 0.5 μL of each primer, 1 μL of PCR mix, and 8 μL of double distilled water (ddH₂O), in a total volume of 20 μL. The cycling conditions for amplification were 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The products were analysed with electrophoresis on 1.0% agarose gel and sequenced by Dalian TaKaRa Company (Dalian,China).

The IFITM3 mRNA levels in the chicken immune organs were detected with SYBR Green real-time fluorescence quantitative PCR. Specific primers were designed (Table 1), and were synthesized by Invitrogen Company. Reaction volumes of 20 μL contained 10 μL of SYBR® Premix Ex Taq™ (2x) (Promega), 0.4 μL of FastStart Universal Probe Master, 0.4 μL of forward primer (10 μM), 0.4 μL of reverse primer (10 μM), 2 μL of cDNA, and 6.8 μL of RNase-free ddH₂O. The PCR cycling conditions were 45 cycles of initial denaturation at 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 34 s. The data were analysed with the ΔCt method using the following formula: target gene expression = 2^(-ΔΔCt).

---

Table 1 – Primer sequences

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences (5’-3’)</th>
<th>Amplification fragments(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFITM3-F</td>
<td>5’ATGCGAGCTTACCTCAGCA3’</td>
<td>342</td>
</tr>
<tr>
<td>IFITM3-R</td>
<td>5’TACAGGGCCGCACAGTGTACAA3’</td>
<td></td>
</tr>
<tr>
<td>IFITM3 (BstZ1)-F</td>
<td>5’ATTCGGCCGAGTACAGTGTACAA3’</td>
<td></td>
</tr>
<tr>
<td>IFITM3 (BstZ1)-R</td>
<td>5’TACAGGGCCGCACAGTGTACAA3’</td>
<td></td>
</tr>
<tr>
<td>IFITM3-F</td>
<td>5’CAGAGCTACCCCTAGCAAC3’</td>
<td>235</td>
</tr>
<tr>
<td>IFITM3-R</td>
<td>5’CTGCCCTCCATAGCCTG3’</td>
<td></td>
</tr>
<tr>
<td>IFα- F</td>
<td>5’ATGCGTGCTGCTGCAAGC-3’</td>
<td>569</td>
</tr>
<tr>
<td>IFα- R</td>
<td>5’TGCCCTGTAGGTGTTGGAAT-3’</td>
<td></td>
</tr>
<tr>
<td>ARV-F</td>
<td>5’GCCTCCGGCATACGAGTTT-3’</td>
<td>204</td>
</tr>
<tr>
<td>ARV-R</td>
<td>5’TAGATCGGCGTCAAATGC-3’</td>
<td></td>
</tr>
<tr>
<td>ARV-F</td>
<td>5’CCATACTGACGGTGAAG-3’</td>
<td>441</td>
</tr>
<tr>
<td>ARV-R</td>
<td>5’TATAGATCGGCGTCAAAT-3’</td>
<td></td>
</tr>
<tr>
<td>β-actin-F</td>
<td>5’CAGAGCTACCCTCAGCAAC3’</td>
<td>138</td>
</tr>
<tr>
<td>β-actin-R</td>
<td>5’TTGCCTGTGAGGTTGTGGAT-3’</td>
<td></td>
</tr>
</tbody>
</table>

Distribution of Ifitm3 in Yellow-Feathered Broilers

and Inhibition of Avian Reovirus Multiplication by Ifitm3

(ΔCt = Ct_{target gene} – Ct_{reference gene}). The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as the reference gene. Statistical differences were determined with t test in SPSS 17.0.

**IFITM3 gene expression in ARV-infected chickens immune organs and CEF**

Thirty 20-day-old yellow-feathered broilers were randomly divided into the control group (five and the experimental group) twenty five. The experimental group chickens were infected with 0.2 mL ARV, and five control chickens were slaughtered at the first day of ARV treatment. Then five infection ducks were slaughtered every after every infection. The thymus, spleen, and bursa of Fabricius were collected from each broiler. The expression of *IFITM3* and *IFNα* and the replication of ARV were detected using reverse transcription (RT)–PCR and SYBR real-time fluorescence quantitative PCR.

CEF grew in six-well tissue-culture plates with MDME cell medium for 37 °C 24 h. CEF cells were collected after inoculation with ARV 0 h, 12 h, 24 h, 48 h, 72 h respectively. The cells were t digested with 0.25% pancreatic enzyme. The total RNA was extracted and the mRNA expression of *IFN*, *IFITM3* and ARV was determined with PCR and SYBR real-time fluorescence quantitative PCR.

**Analysis of IFN sensitivity**

CEF grown in six-well tissue-culture plates were either mock-treated or treated with IFNα (100 IU/ml) for 0.5 h at 37 °C before viral inoculation. Then, these cells were challenged with 0.1 mL of ARV for 24 h and digested with 0.25% pancreatic enzyme. The total RNA was extracted and the expression of *IFITM3* was determined with PCR.

*IFITM3*-directed small interfering RNAs (siRNAs) were designed (F: 5¢-GAGCCUCAGAAACCUCUAAudTdT-3¢ and R: 5¢-AUUAAGGUUUAGGCCUCdTdT-3¢) and transfected into CEF cells. The cells were inoculated with 0.1 mL of ARV for 24 h and then digested with 0.25% pancreatic enzyme. The total RNA was extracted and the mRNA expressions of *IFNα*, *IFITM3* and ARV were detected with PCR and SYBR real-time fluorescence quantitative PCR.

**RESULTS**

**Cloning and analysis of the *IFITM3* gene**

Total RNA was extracted from the spleens of chickens and reversed transcribed to cDNA. PCR with specific primers amplified the 342-bp target gene (Figure 1A). The PCR product was ligated to the linearized pGEM-T Easy cloning vector and competent *E. coli* DH5α cell were transfected with the recombinant plasmid. The recombinant plasmid was then identified with enzymatic digestion. The target gene was 342 bp on 1% agarose gel electrophoresis (Figure 1B), indicating that the cloning vector was successfully constructed. The sequence of *IFITM3* in the yellow-feathered broiler was determined and compared with homologous sequences using BLAST. The sequence of *IFITM3* in the yellow-feathered broiler was high ratio of homology to Gallus *IFITM3* (NCBI LOC422993).

**Figura 1** – Parte A: PCR with specific primers amplified the 342-bp target gene. Parte B: The target gene was 342 bp on 1% agarose gel electrophoresis, indicating that the cloning vector was successfully constructed. Parte C: The amplified fragment was consistent with the anticipated fragment (584 bp).

The *IFITM3* gene sequences of *Homo sapiens*, *Pan troglodytes*, *Macaca mulatta*, *Mus musculus*, *Sus scrofa*, *Bos taurus*, *Equus caballus*, *Felis catus*, and the white-throated sparrow (*Zonotrichia albicollis*) were downloaded from NCBI and their homology was analysed with the DNAMAN software (Figure 2, Table 2). The evolutionary distance between the yellow-feathered broiler and *Zonotrichia albicollis* sequences was smallest, whereas the yellow-feathered broiler sequence was distant from the mammal sequences, and most distant from the human sequence.

**Table 2** – The evolutionary distance of *IFITM3* gene in yellow-feathered broilers and other species

<table>
<thead>
<tr>
<th>Species</th>
<th>Evolutionary distance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Homo sapiens</em></td>
<td>0.059</td>
</tr>
<tr>
<td>Pan troglodytes</td>
<td>0.061</td>
</tr>
<tr>
<td>Macaca mulatta</td>
<td>0.104</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>0.183</td>
</tr>
<tr>
<td>Sus scrofa</td>
<td>0.102</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>0.114</td>
</tr>
<tr>
<td>Equus caballus</td>
<td>0.114</td>
</tr>
<tr>
<td>Felis catus</td>
<td>0.120</td>
</tr>
<tr>
<td>Zonotrichia albicollis</td>
<td>0.209</td>
</tr>
</tbody>
</table>
Amino acid sequence prediction and analysis of the properties of IFITM3

The amino acid sequence of IFITM3 was predicted and the protein properties were analyzed with DNAMAN. The isoelectric point (pI) of IFITM3 is 8.8, and its secondary structure consists of helices, folds, and curls, but predominantly folds and curls (Figure 3A). IFITM3 contains 113 amino acids, and has 20 fewer amino acids than human IFITM3. It contains a CD225 domain (Figure 2B), as does human IFITM3. There are two transmembrane domains, at amino acids 37–65 and amino acids 78–106 (Figure 3C), and three antigenic peptides. The IFITM3 protein of the yellow-feathered broiler is hydrophobic (Figure 3D).

The S-palmitoylation modification sites of IFITM3 in the yellow-feathered broiler were analyzed with the CSS -Pal M 2.0 software. There are four potential palmitoylation sites at cysteines 45, 49, 53, and 89 (Table 3).

Table 3 – The IFITM3 protein of yellow-feathered broilers’ S-palmitoylation modification site

<table>
<thead>
<tr>
<th>Site</th>
<th>Locus</th>
<th>Possibility of palmitoylation modified loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>49</td>
<td>NAFCLGL</td>
<td>0.65</td>
</tr>
<tr>
<td>89</td>
<td>FAFCVGL</td>
<td>0.61</td>
</tr>
<tr>
<td>53</td>
<td>LGLCALS</td>
<td>0.50</td>
</tr>
<tr>
<td>45</td>
<td>FVLCNAF</td>
<td>0.17</td>
</tr>
</tbody>
</table>
Construction of pEASY™-E1-cIFITM3 and prokaryotic expression of the recombinant protein

The recombinant plasmid was amplified and identified with PCR using the T7 primer and target gene primer. The amplified fragment was consistent with the anticipated fragment (584 bp; Figure 1C). The positive plasmid was sequenced by the Dalian TaKaKa Biotechnology Company, confirming the successful construction of the prokaryotic expression vector.

SDS-PAGE showed that the recombinant protein was successfully expressed in the BL21(DE3)pLysS cells, with the expected molecular weight of 11.5 kDa (Figure 4A). The recombinant protein was purified with affinity chromatography using ProteinIso™ Ni-NTA Resin. SDS-PAGE confirmed the successful purification of the recombinant protein (Figure 4B).

Specificity of the anti-cIFITM3 polyclonal antibody

After the immunization program, the anti-rIFITM3 rabbit serum was collected and the antibody titer was determined with ELISA method. The antibody titer reached to 1:256000. The rIFITM3 protein was detected with western blotting, demonstrating that the polyclonal antibody was specific for the rIFITM3 protein (Fig. 4C).

Expression of IFITM3 in immune tissues

The distribution of IFITM3 in the chicken immune organs was examined with immunohistochemistry. IFITM3 was distributed in the thymus (Figure 5A), spleen (Figure 5B), and bursa of Fabricius (Figure 5C). Positive cells were mainly distributed in the medulla of the thymus (Fig. 5D), in the red pulp of the spleen (Fig. 5E), and in the epithelium of the bursa of Fabricius (Fig. 5F). The IFITM3 mRNA levels in 20-day-old yellow-feathered broilers were also investigated with RT–PCR and SYBR–PCR. IFITM3 mRNA was expressed in the three immune organs (Fig. 5G,H), with the highest expression in the bursa.
the thymus (Figure 5D), in the red pulp of the spleen (Figure 5E), and in the epithelium of the bursa of Fabricius (Figure 5F). The IFITM3 mRNA levels in 20-day-old yellow-feathered broilers were also investigated with RT–PCR and SYBR–PCR. IFITM3 mRNA was expressed in the three immune organs (Figure 5G,H), with the highest expression in the bursa.

**IFITM3 expression in ARV-infected CEF cells and yellow-feathered broilers**

After ARV infection 12h and 24h, the mRNA expression levels of IFN-α and IFITM3 in CEF cells was higher than the control group (Figure 6A,B).

To investigate the function of IFITM3 after ARV infection, the mRNA expression levels of IFITM3 and IFN-α in the thymus, spleen, and bursa were determined with RT–PCR and SYBR-qPCR. IFN-α expression was induced by ARV, and then stimulated the expression of IFITM3 early in infection. IFITM3 expression in the thymus (Figure 6C,D) and bursa (Figure 6E,F) peaked on day 3 after ARV infection, and then gradually decreased. In the spleen (Figure 6G,H), IFITM3 began to increase on day 1 after infection, and peaked on day 2. Therefore, ARV stimulated the secretion of IFITM3 in the early stage of ARV infection.

**IFN induces IFITM3 expression to inhibit ARV replication**

Cultured CEFs were infected with ARV. IFN-α (20 ng/mL) was added to the culture fluid of the experimental group and an equal amount of physiological saline was added to the control group. In CEFs with IFN activity, ARV expression peaked at 3 h, and then gradually decreased, whereas the expression of IFITM3
continued to increase gradually (Figure 7 A, B). But ARV expression gradually increased in IFN-null CEFs, and that the expression of IFITM3 peaked at 3 h, gradually decreasing thereafter (Figure 7 C, D).

**RNA interference downregulates the expression of IFITM3 and affects ARV replication**

CEF cells were transfected with siRNA of IFITM3 for 0 or 24 h. The RNA was then extracted and reverse transcribed to cDNA, using β-actin as the internal control. RT–PCR was used to determine the effects of the different periods of IFITM3 interference on ARV expression. When interference at 0 h was compared with no interference at 0 h, neither IFITM3 nor ARV expression changed. After interference for 24 h, IFITM3 expression decreased relative to that in the interference-free control, showing that a low level of RNA interference was achieved (Figure 7 E, F). ARV expression increased when IFITM3 expression was inhibited with IFITM3 interference. With no interference, ARV expression decreased. These results indicate that IFITM3 protein could inhibit replication of ARV.

**DISCUSSION**

There are five members of the IFITM family in humans, IFITM1, IFITM2, IFITM3, IFITM5, and IFITM10, and these proteins are all induced by type I and type II IFNs (Laura et al., 2014). They are involved in many physiological activities, including cell adhesion, cell differentiation, cell signal transduction, antitumor and antiviral activities, and immune surveillance (Perreira et al., 2013). Research recently showed that IFITM was an important antiviral protein, resisting many kinds of viral infections. The replication of influenza virus, West Nile virus, and Dengue virus was significantly inhibited by IFITM3 (Smith et al., 2013). IFITM1 is also an important factor in anti-hepatitis-C-virus infection mechanisms (Canoui et al., 2016). Lu et al. found that the IFITM protein inhibited HIV-1 replication via IFN by uncoating the virus (Lu et al., 2011). Yount et al. showed that the antiviral function of IFITM3 was mediated by the posttranslational S-16 acylation of IFITM3 (Yount et al., 2010). However, the function of IFITM3 in poultry is still unclear.
The IFN-induced expression of the IFITM proteins is ubiquitous. Although IFITM5 is only expressed in bone cells, other IFITM-encoding genes are widely expressed in tissues and organs (Siegrist et al., 2011). IFITM3 is mainly distributed on or in the cell membrane, and it inhibits viral entry into the cell (Bailey et al., 2012). The immune organs (thymus, spleen, and bursa) of birds play important roles in resisting viral infection. Our experimental results found that the IFITM3 gene was expressed in the immune organs of chickens, suggesting that IFITM3 was associated with the chicken’s immune function.

Does IFITM3 suppress the proliferation of chicken viruses? Smith et al. found that the IFITM3 protein of chickens inhibited the replication of ARV (Smith et al., 2013). ARV is one of the most important viruses in poultry production, and understanding the effect of IFITM3 on ARV infection is important for the discovery of new strategies to impede ARV transmission. In this study, we found that ARV activated the expression of IFN-α and IFITM3 in the immune organs (thymus, spleen, and bursa of Fabricius) in 20-day-old yellow-feathered broiler chickens artificially infected with ARV. The expression of IFITM3 was also induced in ARV-infected CEFs. When IFITM3 was strongly expressed after its induction by IFN, the replication of ARV was inhibited, but when the IFITM3 gene was knocked down with shRNA, the replication of ARV was enhanced. Therefore, we consider that the FITM3 protein could inhibit the replication of ARV.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

This work was supported by the Chicken Industry System Program of Fujian China (NO. K83139297) and the National Spark key Program of China (2015GA720001).

COMPLIANCE WITH ETHICAL STANDARDS

All chicks used in the experiments were treated in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of China. The animal protocol used in this study was approved by the Research Ethics Committee of the College of Animal Science, Fujian Agriculture and Forestry University, Fujian, China.

REFERENCES


Chen YX, Welte K, Gebhard DH. Induction of T cell population by antibody to a 16kd human leukocyte surface antigen Immunology 1984;133: 2496-2501.


Erratum

In the article Distribution of Ifitm3 in Yellow-Feathered Broilers and Inhibition of Avian Reovirus Multiplication by Ifitm3 published in the Revista Brasileira de Ciência Avícolas/Brazilian Journal of Poultry Science, v20 (2):305-316, in page 380, 381, 382 and 383 where it was written:

Figure

the correct form is

Figure

http://dx.doi.org/10.1590/1806-9061-2017-0662