Characterization of chicken interferon-inducible transmembrane protein-10

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Abbreviations: CEF, chicken embryonic fibroblast; DMEM, Dulbecco's modified Eagle's medium; cPGC, circulating primordial germ cell; FBS, fetal bovine serum; gPGC, gonadal primordial germ cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; grPGC, germinal ridge primordial germ cell; H-H stage, Hamburger–Hamilton stage; IFN, interferon; IFITM, interferon-inducible transmembrane protein; ISRE, interferon-stimulated response element; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PGC, primordial germ cell; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; VSV-G, vesicular stomatitis virus G protein; SDS, sodium dodecyl sulfate; SSEA-1, stage-specific embryonic antigen-1.

ABSTRACT

Interferon-inducible transmembrane protein (IFITM) family proteins are anti-virus factors. In the present study, we examined the expression pattern of chicken IFITM10 using quantitative reverse transcription-polymerase chain reaction. In adult chickens, IFITM10 levels were markedly lower than those of IFITM3, which exhibits anti-virus activity. On the other hand, IFITM10 was expressed in levels similar to those of IFITM3 in embryonic organs. Primordial germ cells in 2.5-d embryos expressed high levels of IFITM10, which gradually decreased with time. The interferon-α stimulation of embryonic fibroblast cells did not enhance the expression of IFITM10. The forced expression of IFITM10 slightly inhibited the infectivity of the VSV-G-pseudotyped lentiviral vector. Furthermore, cell fusion was inhibited by IFITM10 when HeLa cells transfected with the VSV-G expression vector were treated with low pH buffer. Although it remains unclear whether IFITM10 inhibits virus infections under physiological conditions, these results suggest that chicken IFITM10 exhibits anti-virus activity.

Key words: chicken, IFITM10, interferon, virus prevention

Introduction

Several interferon (IFN)-inducible transmembrane protein (IFITM) family proteins (IFITM1, 2, 3, 5, and 10 for humans, and 1, 2, 3, 5, 6, 7, and 10 for mice) have been identified in humans and mice.[1,2] IFITM1, 2, and 3 block virus replication by preventing the fusion of virus and host cell membranes, thereby restricting numerous pathogenic viruses including influenza A virus and Ebola virus.[1,2] Since influenza viruses prevail on a worldwide scale in chickens, with the potential for a pandemic, chicken IFITMs have recently been studied. To date, IFITM1, 2, 3, 5, and 10 have been detected in chickens. A previous study reported that chicken IFITM3 restricted infections by influenza virus and lyssavirus *in vitro*.[3] We also found that chicken IFITM3 prevented infections by a vesicular stomatitis virus G protein (VSV-G)-pseudotyped lentivirus, which has been used as a viral vector for gene transfer.[4]

In mammals, numerous biological roles have been reported for IFITMs in addition to virus prevention. IFITM1 exerts anti-proliferative effects and facilitates cell adhesion.[5-7] IFITM3 blocks cell proliferation,[8] and IFITM2 induces apoptosis.[9] IFITM3 reduces the endocytosis of transferrin or EGF.[10] In addition, it is reported that IFITM 3 is involved in primordial germ cell (PGC) development in mice.[11,12] IFITM5 is expressed in osteoblasts and may play a role in bone matrix mineralization.[13,14] Most IFITM family genes are located in clusters: for example, within a 70-kb region of mouse chromosome 7 (containing IFITM1, 2, 3, 5, and 6). A recent study indicated that mice lacking this cluster (IFITM1, 2, 3, 5, and 6) exhibited age-related obesity associated with a dysregulated leptin pathway.[15] IFITM10 is outside the cluster in mammals and chickens, being 1.3-1.4 Mb apart on the same chromosome in mice, and the homology of IFITM10 between vertebrates was found to be the highest among IFITMs.[16] On the other hand, the function of IFITM10 has not been elucidated.

In the present study, we analyzed the expression of IFITM10 in adult chickens and embryos, and found that IFITM10, along with IFITM3, was the major IFITM in embryonic organs and PGCs. IFITM10 modestly inhibited VSV-G-mediated cell fusion and the infectivity of a VSV-G-pseudotyped lentiviral vector, suggesting its anti-virus activity.

Materials and methods

Reagents and cells. Chicken IFN-α was purchased from Abcam (Cambridge, UK). An anti-stage-specific embryonic antigen (SSEA)-1 antibody and phycoerythrin-labeled goat anti-mouse IgM antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin and anti-FLAG antibodies were obtained from Medical & Biological Laboratories (MBL, Nagoya, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. The chicken fibroblast cell line DF-1 (CRL-12203) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HeLa and DF-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/mL penicillin G, and 100 μg/mL streptomycin.

Chickens and eggs. Fertilized eggs (White Leghorn) were purchased from Nisseiken (Yamanashi, Japan) or Takeuchi Farm (Nara, Japan), or were obtained in our laboratory from the descendants of those from Nisseiken. Chicken embryonic fibroblasts (CEFs) were obtained using a standard method with trypsin from the eggs that had been incubated at 38°C and 65% humidity with rocking for 10 days. Cells were cultured in DMEM supplemented with 10% FBS and antibiotics, and were used for experiments after 2-3 passages. Organs were isolated from adult chickens and 5.5-d embryos by dissection, cut into small pieces, washed with phosphate-buffered saline (PBS). Tissues from a chicken housed under specific-pathogen-free conditions were kindly provided by Dr. Inayoshi (Nisseiken). All animal experiments were performed according to the ethical guidelines for animal experimentation of Nagoya University.

Isolation of PGCs. Cells expressing SSEA-1 were isolated as PGCs using a fluorescence-activated cell sorter (EPICS ALTRA, Beckman-Coulter, Fullerton, CA, USA or FACSJazz, BD Biosciences, San Jose, CA, USA), as described previously.[17,18] Briefly, fertilized eggs were incubated at 38°C for 55 h (Hamburger–Hamilton (H-H) stages[19] 13–16) and 5.5 days (H-H stages 27–28) to isolate circulating PGCs (cPGCs) and gonadal PGCs (gPGCs), respectively. In order to obtain cPGCs,

blood was drawn with a glass micropipette from embryonal blood vessels, and cells were washed with PBS containing 0.5% FBS. In order to obtain gPGCs, embryonic gonads were isolated with forceps and dispersed in 0.1% trypsin and 0.02% EDTA. Dispersed blood and gonadal cells were incubated with the anti-SSEA-1 antibody for 1 h, and then with the phycoerythrin-labeled goat anti-mouse IgM antibody for 1 h on ice. In order to obtain PGCs in the germinal ridge (grPGCs), the germinal ridges of 3.5-d embryos (H-H stages 18–19) were dissected, treated with 0.1% trypsin plus 0.02% EDTA, and SSEA-1^{+} cells were purified using a similar method to that for gPGCs.

Cloning of chicken IFITM10. In the cloning of chicken IFITM10, cDNAs were obtained from the mRNA of 3-d embryos, and amplified by polymerase chain reaction (PCR) using KOD -Plus- Neo (Toyobo, Osaka, Japan) with the following primers: dir: 5'-cataagcttaccatggacggacggacaggcagc-3'; rev: 5'-catgaattctcgtaatcggtgaggggtaccgcagg-3' (underlines indicate restriction sites for cloning). Amplified DNA was inserted into the *Hin*dIII/*Eco*RI sites of a p3xFLAGCMV14 plasmid (Sigma-Aldrich). Chicken IFITM10 lacking an N-terminal region was amplified by PCR using the primers: dir: 5'-aaaaagcttaccatgaaccccaccaccgtcatcgagatct-3'; rev: 5'-catgaattctcgtaatcggtgaggggtaccgcagg-3' (underlines indicate restriction sites for cloning) and cloned into p3xFLAGCMV14. As the control, human IFITM3 was also cloned using cDNAs from HeLa cells as a template with the following primers; dir: 5'-aaagaattcaccatgaatcacactgtccaaaccc-3'; and rev: 5'-aaaggatcctccataggcctggaagatcag-3' (*Eco*RI and *Bam*HI sites are underlined). Amplified DNA was cut with *Eco*RI/*Bam*HI and cloned into p3xFLAGCMV14. Nucleotide sequences were determined in Nagoya University Center for Gene Research using ABI PRISM 3100 (Termo Fisher Scientific, Yokohama, Japan).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Real-time PCR was performed using LightCycler (Roche Diagnostics, Mannheim, Germany) or LightCycler 96 (Roche Diagnostics). Total RNAs were isolated using ISOGEN II (Nippon Gene, Tokyo, Japan), reverse transcribed by ReverTra Ace (Toyobo) with an oligo-dT primer, and subjected to qPCR using SYBR Green I dye (Thunderbird qPCR Mix, Toyobo). Due to the small number of PGCs

available, the ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI, USA) and ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo) were used for the extraction of total RNA and reverse transcription for PGCs. LightCycler amplification involved denaturation at 95°C for 60 s, followed by the amplification of target DNA for 40 cycles (95°C for 3 s, 60°C for 10 s, and 72°C for 30 s).

The amount of each cDNA was first measured with LightCycler Software (Roche Diagnostics) based on the standard curve obtained using a corresponding plasmid after linearization, and expression levels were then normalized by calculating the ratio of the mRNA of interest to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Standard plasmid DNAs, which contained the cloned cDNA of GAPDH, IFITM2, 3[4)] and 10 were cut using a restriction enzyme and purified by a nucleotide removal kit (Qiagen, Tokyo, Japan).

cDNA was amplified by PCR with the following primers: IFITM2, dir: 5'-aggtgagcatcccgctgcac-3', rev: 5'-accgccgagcaccttccagg-3'; IFITM3, dir: 5'-tcacggcccatctgatcaac-3', rev: 5'-gggtccaatgaattcggggt-3'; IFITM10, dir: 5'-attcgctgaaagtccgggat-3', rev: 5'-gggctgagctggtgatgtta-3'; GAPDH, dir: 5'-gggcacgccatcactatc-3', and rev: 5'-gtgaagacaccagtggactcc-3'. Regarding chicken IFITM10, longer and shorter variants appeared on the database. Putative exon 2 was deleted in the latter variant. The primer set of qRT-PCR for IFITM10 corresponded to the sequence in the C-terminal half of the CD225 domain, and this primer set detected the longer and shorter variants. In order to detect the longer variant, an N-terminal primer set was designed from the sequence in putative exon 2 that was deleted in the shorter variant, dir: 5'-gacaaccagagggacagcc-3'; rev: 5'-atcctgcgtcctctccgt-3'.

Induction of IFITM10 by IFN-α. CEFs were seeded at 1.0 x 10^5 cells per well on a 24-well plate (Nunc, Roskilde, Denmark) and cultured for 24 h. Cells were cultured for an additional 18 h in the presence of IFN-α (200 ng/mL). RNAs were then isolated using ISOGEN II, and IFITM10 mRNA was quantified by qRT-PCR.

DF-1 cells stably expressing IFITM10. DF-1 cells were seeded at 1.0 x 10^5 cells per well on a 24-well plate, and transfected with the IFITM10 expression vector, which had been linearized by cutting with *Sca*I using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48

h, 400 μg/mL G418 was added, and surviving cells were used as mixtures (not cloned). As the control, an empty p3xFLAGCMV14 vector was similarly transfected.

VSV-G-pseudotyped lentiviral vector production. Lentiviral vectors (pSicoR, which expressed eGFP under the control of CMV promoter) were produced as described previously.[4] The viral titer was determined with HeLa cells as the percentage of cells expressing the eGFP gene.

Estimation of infectivity. DF-1 cells were seeded at 5.0 x 10^4 cells per well on a 24-well plate. After 24 h, the viral vector was infected to the cells at a MOI (multiplicity of infection) = 0.1. Infectivity was measured 9 h post-infection by quantifying viral cDNA in the cytosol that had been reverse transcribed by viral reverse transcriptase, as described previously.[4]

VSV-G-mediated cell fusion. HeLa cells (1 x 10^5 cells/well) were inoculated in a 24-well tissue culture plate, and cultured for 24 h in DMEM. Cells were transfected with 0.5 μg of the IFITM10 expression vector by Viafect (Promega) as recommended by the supplier. After an overnight culture, cells were transfected with 0.3 μg of the VSV-G expression vector (Invitrogen) by Viafect. Cells were cultured for another 24 h, then stained with 500 ng/mL Calcein-AM (Dojindo Laboratories, Kumamoto, Japan) in Opti-MEM at 37°C for 15 min. Cells were washed with PBS and then incubated with fusion buffer (10 mM HEPES and 10 mM MES, pH 5.5) for 1 min. After removing the fusion buffer, 500 μl of DMEM was added, and cells were left to stand at 37°C for 15 min. Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min, then analyzed by In Cell Analyzer 2000 (GE Healthcare, Tokyo, Japan) or Biozero (Keyence, Osaka, Japan). The surface area covered by fused cells or all cells was assessed using ImageJ software[20] and shown as "% fusion area", which was calculated by the equation (surface area covered by fused cells) / (surface area covered by all cells) x 100.

Western blotting. Cells were lysed with sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes and probed with a specific

antibody, followed by a peroxidase-labeled anti-IgG antibody. The membrane was developed with ECL plus (Perkin Elmer, Yokohama, Japan), and analyzed by LAS-3000 mini (Fuji Film, Tokyo, Japan), as described previously.[21] Band intensity was evaluated using ImageJ.

Results

Cloning of chicken IFITM10

Two variant forms of IFITM10 appeared on the database (XM_001234445.3 and XM_001234445.2) based on in silico prediction; the shorter variant lacked nucleotides 27 to 156 (putative exon 2), possibly due to alternative splicing (Supplementary Fig. 1A). In the cloning of IFITM10, the primer set was designed from the database, and cDNA was amplified and cloned into the p3xFLAGCMV14 vector. Ten colonies were picked up and sequenced, however, only the shorter variant was picked up in our experiment. In order to establish whether the longer variant was transcribed, another primer set was designed in exon 2. RT-PCR revealed that no cDNA was amplified from mRNA of 5.5-d embryonic and adult organs (Supplementary Fig. 1B), suggesting that the longer variant was not transcribed. The DNA sequence of IFITM10 elucidated by us completely matched that of the database. Since only the shorter variant was detected in our analysis, amino acid numbers were hereafter indicated based on this form. The amino acid sequence of IFITM10 is known to be conserved well, whereas that of IFITM1, 2, and 3 vary between organisms. The putative CD225 domain (amino acids 126 to 187 in chickens) containing intramembrane regions 1 and 2 and the conservative intracellular loop was detected by amino acid alignment (Supplementary Fig. 2A). Chicken IFITM10 showed high homologies in the N-terminal half of the CD225 domain (intramembrane region 1) and its N-terminal side sequence (amino acids 80 to 126) to those of humans and mice. The intramembrane region 2 and conserved intracellular loop were similar between humans and chickens, but not mice. On the other hand, the N-terminal sequence of 35 amino acids was similar between humans and mice, but different from that of chickens.

The expression of IFITM10 in adult chickens and embryos

We previously examined the expression of IFITM1, 2, 3, and 5 and found that

IFITM3 expression was highest in both adult and embryonic chicken organs and that its level was much higher in adult.[4] The expression of IFITM10 in adult chicken organs was examined using qRT-PCR (Fig. 1). Its expression was detected in most of the organs analyzed, except for the liver and testis. Since the efficiency of reverse transcription may slightly differ for each gene, the precise comparison of their expression levels is difficult; however, our results showed that the copy numbers of IFITM10 mRNA ($1.07 \pm 0.80 \times 10^{-2}$ versus GAPDH in the oviducts, which showed the strongest expression of IFITM10), measured by qRT-PCR, were markedly lower than those of IFITM3 in the lungs (1.12 ± 0.32 versus GAPDH, data not shown), which was highest among IFITMs in adult organs.[4] Organs from a chicken housed under specific-pathogen-free conditions showed similar IFITM10 expression levels (data not shown). Collectively, these results indicate that the expression level of IFITM10 in the adult chicken was very low.

The expression of IFITM10 in embryos was also examined (Fig. 2). Several organs were isolated from 5.5-d embryos, and the expression of IFITM10 was analyzed by qRT-PCR. IFITM10 was expressed in all of the organs analyzed, and was strongly expressed in the gonads. The expression of IFITM3, a major IFITM species in adult organs, was weaker in embryonic organs,[4] although it was not possible to make exact comparisons between adult chickens and embryos because the organs analyzed were different. On the other hand, the expression of IFITM10 appeared to be stronger in embryonic organs (compare Fig. 1 and 2). Thus, the expression of IFITM3 and 10 measured by qRT-PCR was similar in embryonic organs. Among the embryonic organs tested, the gonads showed the highest expression levels of IFITM10; therefore, we examined the expression of IFITM10 in PGCs (Fig. 3). The mRNA level of IFITM10 measured by qRT-PCR in the early stage of PGCs (cPGCs) was highest, then gradually decreased, as observed with IFITM3.[4]

IFITM10 expression was not enhanced by IFN-α in CEFs

The antiviral effects of IFN-α were partly mediated by its inducible genes including IFITMs. Human IFITM1, 2, and 3 are known to be induced by an IFN stimulation,[1,4] while the induction of IFITM3, but not IFITM1 or 2, was strong in mice.[22] Unstimulated CEFs expressed very low levels of IFITM10 (Fig. 4A). Its expression level was approximately 2% that in the embryonic gonads. The stimulation

of CEFs with IFN-α slightly enhanced the expression of IFITM10: an approximately 1.4-fold induction, but the difference was not statistically significant. In CEFs, the major IFITMs were IFITM2 and 3.[4] The expression of IFITM2 could be induced almost twenty-fold by this concentration of IFN-α (Fig. 4A), as reported.[4] Thus, this result suggests that IFITM10 did not strongly participate in IFN-induced antiviral activity in chickens. In IFITM2 and 3, the putative IFN-stimulated response element (ISRE) was detected around the translation initiation site (Fig. 4B). On the other hand, a typical ISRE was not observed in the 5' upstream region of the IFITM10 gene, except for one at approximately -400 from the initiation codon. Although putative ISRE was not observed within -500 bp region of IFITM1 or 5, these were modestly induced by IFN-α. Therefore we could not rule out the possibility that unknown mechanisms regulated the expression of IFITM1, 5 in the presence of IFN-α.

Chicken IFITM10 repressed the infectivity of the VSV-G-pseudotyped lentiviral vector in DF-1 cells

Mammalian IFITM3 strongly restricts infections by various viruses that infect through late endosomes, and IFITM1 blocks infections through early endosomes.[1] We previously demonstrated that chicken IFITM3 restricted infections by VSV-G-pseudotyped lentiviral vectors using DF-1 cells,[4] as was reported with influenza A virus and lyssavirus.[3] Therefore, we investigated whether chicken IFITM10 inhibited infections by the VSV-G-pseudotyped lentiviral vector. Since chicken DF-1 cells expressed very low levels of IFITM10 (less than 10^{-5} of GAPDH), we established a stable cell line that expressed IFITM10. The expression level of IFITM10 was similar to cPGCs and endogenous IFITM3 of DF-1 in terms of mRNA copy numbers (Fig. 5A). As shown in Fig. 5B, the infectivity of the VSV-G viral vector was slightly reduced in the stable cell line; however, this difference was very small. These findings indicated the potential of chicken IFITM10 for virus prevention; however, it is not clear whether IFITM10 plays roles in preventing viruses under physiological conditions in chickens because its expression level was markedly lower than that of IFITM3, except in embryonic organs, and its expression was not induced by IFN.

Overexpression of chicken IFITM10 blocked VSV-G-mediated cell fusion

Viral envelope proteins induced cell fusion when artificially expressed on the cell surface, and it was reported that IFITMs blocked the fusion.[23] The inhibition of envelope-induced cell fusion, which is often called a syncytia formation assay, predicts the inhibitory activities of IFITMs against viruses, although some inconsistent findings have been reported for unknown reasons.[23] Therefore, we investigated whether IFITM10 blocked cell fusion mediated by VSV-G because VSV-G has been shown to strongly facilitate cell fusion in a short treatment with an acidic buffer.[24-26] IFITM10 and human IFITM3 were transiently expressed in HeLa cells (Fig. 6A). The migration of IFITM10 was slower (28 kD) than the calculated size (24 kD), which was also observed for hIFITM3 (apparent size: 22-24 kD for calculated 17.5 kD), and this may have been due to modifications such as phosphorylation, palmitoylation, and ubiquitination.[27] After the cell surface expression of VSV-G followed by a short treatment with an acidic buffer, cells fused with each other and formed large syncytia (Fig. 6B, C), as expected.[24-26] Cells expressing human IFITM3 showed lower levels of cell fusion, as reported previously.[23] Furthermore, chicken IFITM10 strongly blocked cell fusion. Expression levels measured by the band intensities of major bands (Fig. 6A) suggested that the expression level of IFITM10 was 1.6-fold higher than that of human IFITM3, which may partly explain the stronger inhibition by IFITM10.

The N-terminal regions before the CD225 domain are variable in the IFITM family and IFITM10 has a longer sequence than those of other IFITMs (Supplementary Fig. 2B). In order to examine the possible role of the N-terminal region, the truncated form of IFITM10 lacking the first 105 amino acids (ΔN) was expressed and the inhibition of VSV-G-mediated cell fusion was examined. The IFITM10 (ΔN) construct strongly inhibited cell fusion (Supplementary Fig. 3), suggesting that this region is not essential for IFITM10 to inhibit cell fusion.

Discussion

IFITM1, 2, and 3 are antiviral cell-intrinsic restriction factors.[1,2] IFITM5 is mainly distributed in osteoblasts, which suggests the involvement of this IFITM species in bone formation. On the other hand, the physiological function of IFITM10 has not yet been elucidated. In the present study, we demonstrated that IFITM10 prevented

VSV-G-mediated cell fusion and its inhibition was similar to that of human IFITM3 (Fig. 6). We also found that IFITM10 weakly inhibited the infection of VSV-G-pseudotyped lentiviral vectors (Fig. 5).

A potential underlying mechanism for the prevention of virus infections is the inhibition of fusion between endosomes and viral membranes. This is consistent with the results of the present study, but does not necessarily mean that IFITM10 has physiological functions such as virus prevention because a typical ISRE was not detected in the promoter region of IFITM10 and it was not induced by IFN (Fig. 4). Furthermore, the copy number of IFITM10 in adult chickens was markedly lower than that of IFITM3. However, we cannot rule out the possibility that low IFITM10 levels have some functions in chickens, similar to IFITM5, of which knockout caused reproductive disorders in spite of apparent osteoblast-specific expression in mice.[14)]

In the present study, we identified IFITM10 as a major IFITM species in chicken embryos, but it is not clear whether IFITM10 prevents virus infections in embryos because maternally transferred IgY is considered to play major role in the prevention of progenies from the infection. In addition to embryonal organs, cPGCs expressed relatively high levels of IFITM10, which gradually decreased with the development of PGCs in chickens, similar to IFITM3.[4)] In mice, IFITM3 was also strongly expressed in PGCs[12)]; however, its physiological role in PGC development currently remains unclear because mice lacking IFITM3 or the IFITM locus (IFITM1, 2, 3, 5, and 6) show normal fertility.[28)] Thus, it is interesting to address whether IFITM10 is involved in PGC regulation in chickens. Further investigation is necessary to elucidate the physiological roles of IFITM10 in chickens.

Author contributions

SI and KN designed the experiments; YO, SK, HK performed the experiments; YO, SK, HK, SI, and KN analyzed the data; YO, SI and KN wrote the manuscript.

Acknowledgments

We would like to thank the National Bio-Resource Project of the MEXT, Japan (Nagoya University Graduate School of Bioagricultural Science, Avian Bioscience Research Center) for their support of the PGC experiments, and Dr. Y.

Yoshida (Innovative Research Center for Preventive Medical Engineering, Nagoya University) for the use of FACS Jazz.

Disclosure statement

The authors have declared that no conflicts of interest exist.

Funding

This work was partly supported by JSPS KAKENHI Grant Number 26289312.

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Figure legends

Fig. 1. Expression of chicken IFITM10 in various adult chicken organs.
Adult chicken organs were subjected to qRT-PCR. Expression levels are shown as a ratio to that of GAPDH. Data are the mean ± standard error of six different chickens (three male and three female), except for the ovary, oviduct, and testis.

Fig. 2. Expression of chicken IFITM10 in various embryonic organs.
Organs from two 5.5-d embryos were pooled and subjected to qRT-PCR. Note that several organs including the lungs and spleen did not develop well at this stage, thus, were excluded from the analysis. Data are the mean ± standard error of three independent experiments. The arrow indicates the level of IFITM3 in the embryo gonads ($2.89 \pm 0.11 \times 10^{-2}$ versus GAPDH).

Fig. 3. Expression of chicken IFITM10 in PGCs of different developmental stages.
PGCs were sorted from blood (cPGC), germinal ridges (grPGC), and gonads (gPGC), and subjected to a qRT-PCR analysis. SSEA-1$^-$ cells were simultaneously sorted from germinal ridges or gonads and used for somatic cells (soma). Data are the mean ± standard error of three independent experiments.

Fig. 4. Lack of the induction of chicken IFITM10 by IFN-α in CEFs.
(A) CEFs were stimulated with IFN-α and the expression levels of IFITM10 and 2 were analyzed by qRT-PCR. Data are the mean ± standard error of three independent experiments. *, $p < 0.05$ by the Student's *t*-test; NS, not significant. (B) The location of putative ISRE predicted by TFBIND (http://tfbind.hgc.jp/). The calculated scores are shown. The magnitude of induction by IFN-α in our previous study[4)] is also shown on the right. CDS, coding sequence.

Fig. 5. Inhibition of VSV-G-pseudotyped lentiviral vector infection by IFITM10.
(A) Establishment of stable DF-1 cells that expressed IFITM10. Expression levels were evaluated by qRT-PCR. Data are the mean ± standard error of three independent experiments. The arrowhead indicates the IFITM10 level of cPGCs. An empty vector was used as the control vector. (B) The infectivity of the VSV-G-pseudotyped virus.

Data are the mean ± standard error of six independent experiments. *, $p < 0.05$ by the Student's *t*-test.

Fig. 6. Inhibition of VSV-G-mediated cell fusion by IFITM10.
(A) Confirmation of IFITM expression. Expression levels were examined using a C-terminal FLAG tag. The relative intensities of the major bands were measured and shown on the bottom after normalization with β-actin intensity. (B) Cell morphology before and after a treatment with an acidic fusion buffer. Broken lines indicate the boundary of fused and non-fused cells. Bar, 50 μm. (C) Quantification results for cell fusion. Data are the mean ± standard error of triplicate cultures. *, $p < 0.05$ by the Student's *t*-test.

Supplementary Fig. 1. IFITM10 variants on the database and their expression analysis.
(A) Structure of the IFITM10 gene on the database and location of primers used for RT-PCR. The numbers indicate nucleotide numbers. Pair 1, primers for both variants; pair 2, primers for the long variant. (B) RT-PCR analysis of IFITM10 variants. cDNA was amplified by indicated primers (40 cycles of amplification). The lack of longer variant expression was also shown by qRT-PCR (data not shown).

Supplementary Fig. 2. Comparison of amino acid sequences of IFITMs.
(A) Comparison of chicken and mammalian IFITM10. Open and closed triangles show putative ubiquitination (K) and palmitoylation (C) sites in chicken IFITM10, respectively. IM, intramembrane domain; CIL, conserved intracellular loop. (B) Comparison of chicken IFITMs. Analyzed by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Supplementary Fig. 3. The N-terminal region of IFITM10 was not essential for the inhibition of VSV-G-mediated cell fusion.
(A) Schematic representation of IFITM10 lacking the N-terminal region. The numbers indicate amino acid numbers for the short variant in Supplementary Fig. 1. FL, full length IFITM10. (B) Confirmation of IFITM expression. Expression levels were examined using a C-terminal FLAG tag. The relative intensities of the major bands were measured and shown on the bottom after normalization with β-actin intensity. (C)

Fluorescent images before and after the treatment with the fusion buffer are shown. Broken lines indicate the boundary of fused and non-fused cells. Bar, 50 μm. (D) Quantification results for cell fusion. Data are the mean ± standard error of three independent experiments. *, $p < 0.05$ by the Student's *t*-test; NS, not significant.