



# Knock-in of the duck retinoic acid-inducible gene I (*RIG-I*) into the *Mx* gene in DF-1 cells enables both stable and immune response-dependent RIG-I expression

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## ABSTRACT

Waterfowls, such as ducks, are natural hosts of avian influenza virus (AIV) and can genetically limit the pathogenicity. On the other hand, some AIV strains cause severe pathogenicity in chickens. It is suggested that differences in the pathogenicity of AIV infection between waterfowls and chickens are related to the expression of retinoic acid-inducible gene I (*RIG-I*), a pattern recognition receptor that chickens evolutionally lack. Here, we knocked-in the duck *RIG-I* bearing the T2A peptide sequence at the 3' region of the *Mx*, an interferon-stimulated gene (ISG), in chicken embryo fibroblast cells (DF-1) using the precise integration into target chromosome (PITCh) system to control the duck *RIG-I* expression in chickens. The expression patterns of the duck *RIG-I* were then analyzed using qPCR. The knocked-in DF-1 cells expressed *RIG-I* via the stimulation of IFN- $\beta$  and poly(I:C) in a dose-dependent manner. Moreover, poly(I:C) stimulation in the knocked-in DF-1 cells upregulated *RIG-I*-like receptor (RLR) family signaling pathway-related genes *IFN- $\beta$* , *OASL*, and *IRF7*. The IFN- $\beta$ -dependent expression of *RIG-I* and upregulation of *IFN- $\beta$*  in the poly(I:C) stimulation demonstrated a positive-feedback loop via *RIG-I*, usually evident in ducks. Overall, this novel strategy established *RIG-I*-dependent immune response in chickens without overexpression of *RIG-I* and disruption of the host genes.

## 1. Introduction

Retinoic acid-inducible gene I (*RIG-I*) is a pattern recognition receptor belonging to *RIG-I*-like receptors (RLRs), which recognize viral RNA including avian influenza virus (AIV) in the cytoplasm and induce the expression of type-I interferons (IFNs) and cytokines [1–3]. It is suggested that differences in the pathogenicity of avian influenza virus (AIV) infections between waterfowls and chickens are related to the expression of *RIG-I*. Waterfowls, like ducks, are natural hosts of AIVs [4]. Although highly pathogenic AIV (HPAIV) infection is lethal for chickens, ducks can genetically limit the pathogenicity [5]. Chickens evolutionally lack *RIG-I*, and overexpression of the duck *RIG-I* in chicken embryo fibroblast cells (DF-1) results in reductions in the viral titers of AIV strains and upregulation of IFN- $\beta$  and interferon-stimulated gene

(ISG) expression during infection [6]. These previous studies suggest that duck *RIG-I* can enhance host immune resistance against AIV infection in chickens.

There are two problems that should be considered when trying to produce transgenic chickens expressing duck *RIG-I*. First, the expression patterns should be controlled; an AIV infection-dependent expression system of duck *RIG-I* should be established. Constant and high expression of duck *RIG-I* in transgenic chickens may lead to an inflammatory response in non-infected chickens. Therefore, Xiao and colleagues analyzed the expression activity of duck *RIG-I* promoters in DF-1 to address this problem [7]. Second, random integration of duck *RIG-I* into the chicken genome can cause host gene disruption and unstable expression. In random integrations, the location(s) of the inserted gene of interest (GOI) is/are not controlled and thus, the GOI can be inserted

**Abbreviations:** *RIG-I*, retinoic acid-inducible gene I; IFNs, interferons; AIV, avian influenza virus; ISG, interferon-stimulated gene; RLR, *RIG-I*-like receptor; LPAIV, low pathogenic avian influenza virus; HPAIV, highly pathogenic avian influenza virus; ISRE, IFN-stimulated response element; MMEJ, microhomology-mediated end-joining; PITCh, precise integration into target chromosome; DSB, DNA double-strand break; OASL, 2'-5'-oligoadenylate synthase-like protein; IRF7, interferon regulatory factor 7.

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anywhere into the host gene. Furthermore, the GOI can be silenced after random integration using viral vectors. For these reasons, the expression patterns and location of integration of the duck *RIG-I* in the chicken genome should be strictly controlled.

Here, we attempted to establish a strategy for controlling the expression of *RIG-I* via activation of the host gene promoter through gene targeting. As a candidate promoter, we selected the promoter of the interferon-induced GTP-binding protein gene *Mx*. The chicken *Mx* promoter possesses an IFN-stimulated response element (ISRE) sequence, which is activated by interferon stimulation [8]. A rapid increase in *Mx* has been observed in DF-1 cells following infection with both HPAIV and low pathogenic AIV (LPAIV) [9]. Therefore, the chicken *Mx* promoter activity is pertinent for controlling *RIG-I* expression depending on the immune response. On the other hand, as a method for gene targeting, the precise integration into target chromosome (PITCh) system was used to integrate the duck *RIG-I* into the downstream region of the *Mx* gene. The PITCh system is a knock-in strategy that uses microhomology-mediated end-joining (MMEJ), a DNA double-strand break (DSB) repair pathway via annealing of short homologous sequences (5–25 bp) [10,11]. Compared to a knock-in system using homologous recombination, the PITCh system enables both easy and highly efficient integration of a gene of interest into the target region.

In the present study, we integrated the duck *RIG-I* containing the T2A sequence in the 5' region into the downstream region of the open reading frame of the *Mx* gene using DF-1 cells. Expression patterns of the duck *RIG-I* were analyzed after stimulation of both IFN- $\beta$  and poly(I:C), a *RIG-I* ligand, using real-time qPCR. Additionally, the expression analyses of the host innate immune response-related genes were also performed using the real-time qPCR under poly(I:C) stimulation.

## 2. Materials and methods

### 2.1. Construction of donor vector and CRISPR gRNA vectors

To integrate the duck *RIG-I* gene into the chicken genome, a donor vector was constructed. A schematic diagram for the construction of the donor vector is shown in Fig. S1. The nucleotide sequences of the primers used are shown in Table S1. The CRIS-PITCh v2 vector was kindly provided by Dr. Tetsushi Sakuma (Hiroshima University, Japan). An artificial gene, which contains the 3' region and UTR of the chicken *Mx* gene (ENSGALT00000025999.7), used as a template, was synthesized by Eurofins Genomics (Japan). In the synthesized gene, some bases were replaced so as not to transform the amino acid sequence of the *Mx* protein to avoid DSB after the insertion of donor fragments into genome DNA. The nucleotide sequence of the synthesized gene is shown in Fig S2 (nucleotide 44–65 and 2931–3277). To amplify the puromycin resistance gene via PCR, pApo-EF1 $\alpha$  Pur DNA (TaKaRa Bio, Shiga, Japan) was used. Fragments of the artificial gene, puromycin resistance gene, and CRIS-PITCh v2 vector were amplified by PCR using the primers A–B, C–D, and E–F, respectively. The PCR reactions were performed under the following conditions: 30 cycles of 98 °C for 10 s, 65 °C for 5 s, and 68 °C for 10 s (PCR reactions used primers A–B and C–D) or 25 s (the reaction used primer E–F). The fragments were then assembled using an In-fusion™ HD cloning Kit (TaKaRa Bio, Shiga, Japan). In the second reaction, the assembly of the constructed vector and duck *RIG-I* gene was performed. A plasmid containing a clone of the duck (*Anas platyrhynchos*) *RIG-I* gene (NM\_001310380) was kindly provided by Dr. Takehiko Saito (National Institute of Animal Health, Japan). The inverse PCR was performed using the primers G and H, with the constructed vector under the following conditions: 30 cycles of 98 °C for 10 s, 60 °C for 5 s, and 68 °C for 1 min. On the other hand, the PCR reactions to amplify duck *RIG-I* fragments were performed using primers I–J. The T2A sequences were included in the primers G–I. The PCR conditions were 30 cycles of 98 °C for 10 s, 65 °C for 5 s, and 68 °C for 15 s. In these reactions, the KOD One® PCR Master Mix (Toyobo Co. Ltd., Osaka, Japan) was used as a DNA polymerase. Then, the fragments were

assembled using the In-fusion™ HD cloning Kit (TaKaRa Bio, Shiga, Japan), and the donor vector was established. The nucleotide sequence of the donor vector was analyzed with 11 different primers (donor seq primer 1–11), the nucleotide sequences of which are shown in Table S1, using a SeqStudio™ genetic analyzer (Thermo Fisher Scientific, Waltham, Massachusetts).

The pX330-U6-Chimeric BB-CBh-hSpCas9 vector (Addgene #42230) was used as the genome-editing tool. Nucleotide sequences of the DNA oligos used for the construction of gRNA vectors are shown in Table S1. For the construction of a gRNA vector for *Mx*, two oligos (*Mx* sense and *Mx* antisense oligos) were used. The target site of gRNA was designed using CRISPRdirect software (<http://crispr.dbcls.jp>) [11]. On the other hand, a gRNA for the donor vector was constructed as shown in a previous study [12], and donor sense and donor antisense oligos were used. After the construction of vectors, the nucleotide sequences in the gRNA sites were revealed using the pX330 sequencing primer (shown in Table S1).

### 2.2. Single-strand annealing (SSA) assay

To evaluate the cleavage activity of the constructed gRNA vector for *Mx* gene, a SSA assay was performed. The SSA assay is a plasmid-based assay that uses a reporter vector containing target site sequence between inactivated luciferase gene fragments. Once a DSB occurs at the target site in the reporter vector, active luciferase is constructed via single-strand annealing between homologous regions of inactivated luciferase gene fragments. Therefore, the cleavage activity of gRNA vector can be evaluated by chemiluminescence. In this study, the pGL4-SSA vector (Addgene #58227) was used, and details of protocols for the SSA assay using this vector have been described previously [13]. Briefly, to construct the reporter vector including the *Mx* nucleotide sequence, the two DNA oligos (SSA sense or antisense oligos) were annealed. The nucleotide sequences of the oligos are shown in Table S1. Then, the annealed oligo was inserted into the pGL4-SSA vector. The SSA reporter vector, the gRNA vector for *Mx*, and the reference vector were transfected into HEK293 cells, which were seeded at  $5 \times 10^4$  cells/well in 96-well plates, at 100 ng, 400 ng, and 20 ng per well, respectively. As a negative control, the mock vectors that inserted non-specific oligos into gRNA vectors or SSA reporter vectors were used. In this transfection, the FuGENE® HD Transfection Reagent (Promega, Madison, Wisconsin) was used. The HEK293 cells were then cultured for 24 h, and the cleavage activity of the gRNA vector was analyzed using the Dual-Luciferase® reporter assay system (Promega, Madison, Wisconsin). The reporter activity was measured by an ARVO X4 plate reader (PerkinElmer, Waltham, MA, USA).

### 2.3. *RIG-I* knock-in DF-1 cells and cell cloning

The DF-1 cells were obtained from the American Type Culture Collection (ATCC® CRL-12203™), and the HEK293 cells were also obtained from the American Type Culture Collection (ATCC® CRL-1573™). DF-1 cells were cultured in KnockOut™ DMEM (Thermo Fisher Scientific, Waltham, Massachusetts) with 10 % fetal bovine serum (FBS) and 1 % GlutaMAX™ (Thermo Fisher Scientific, Waltham, MA, USA) at 38 °C with 5 % CO<sub>2</sub> and 3 % O<sub>2</sub>. The HEK293 cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) with 10 % FBS at 37 °C with 5 % CO<sub>2</sub>.

To obtain *RIG-I* knocked-in DF-1 cells, 0.8  $\mu$ g of the gRNA vectors and the donor vector were integrated into DF-1 cells, which were seeded at  $2.5 \times 10^5$  cells/well in 6-well plates and cultured for 24 h, using the Lipofectamine® 3000 Reagent (Thermo Fisher Scientific, Waltham, Massachusetts). The DF-1 cells were treated with puromycin at 0.6  $\mu$ g/mL. After puromycin treatment, the DF-1 cells were cloned using the limited dilution method. Screening of the cloned DF-1 cells was performed by PCR using F1–donor seq primer 2, F2–R1, and F1–R1, whose nucleotide sequences are shown in Table S1. The PCR reactions were

performed using KOD One® PCR Master Mix (Toyobo Co. Ltd.) under the following conditions: 35 cycles of 98 °C for 10 s, 65 °C for 5 s, and 68 °C for 1 s. The nucleotide sequences in the insertion sites of screened DF-1 cells were analyzed using F2 and F3 (Table S1), and chromatograms were inspected in the Snap Gene® Viewer.

#### 2.4. Poly(I:C) and chicken interferon- $\beta$ stimulation

The DF-1 cells, which were seeded at  $2 \times 10^5$  cells/well in 12-well plates and cultured for 24 h before stimulation, were treated with recombinant chicken interferon- $\beta$  protein (His tag) (ab239440; Abcam Cambridge, UK) at a dose of 100 or 1000 ng/mL for 4 h. As a negative control, non-stimulated DF-1 cells were used. The DF-1 cells were seeded under the same conditions and were used for transfection of poly(I:C) using the Lipofectamine® 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) at a dose of 0.02 or 0.2  $\mu$ g/mL for 6 h. In this analysis, the short-length Poly(I:C) (InvivoGen, San Diego, California), with an average size of 0.2–1 kb, was used.

#### 2.5. Real-time PCR (qPCR) and RT-PCR

The total RNA was isolated using the FastGene™ RNA Premium Kit (NIPPON Genetics Co., Ltd, Tokyo, Japan). In this cDNA synthesis, the SuperScript IV reverse transcriptase (Thermo Fisher Scientific) was used. For qPCR, a StepOne real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used with the KOD SYBR qPCR mix (Toyobo Co. Ltd.). The primers used in this qPCR are shown in Table S2. The qPCR conditions were 50 cycles of 98 °C for 10 s, 60 °C or 68 °C for 10 s, and 68 °C for 30 s. The qPCR reaction was performed in technical triplicates. The relative expression levels were calculated using the  $\Delta\Delta Ct$  method [14]. The expression levels of each target gene were normalized to that of  $\beta$ -actin.

For RT-PCR, the primers RT-F and RT-R, whose nucleotide sequences are shown in Table S1, were used. RT-PCR was performed using KOD One® PCR Master Mix (Toyobo Co. Ltd.) under the following conditions: 30 cycles of 98 °C for 10 s, 65 °C for 5 s, and 68 °C for 1 s. cDNA derived from 100 ng of total RNA was used as template for RT-PCR.

### 3. Results

#### 3.1. Construction of vectors for knock-in using PITCh methods

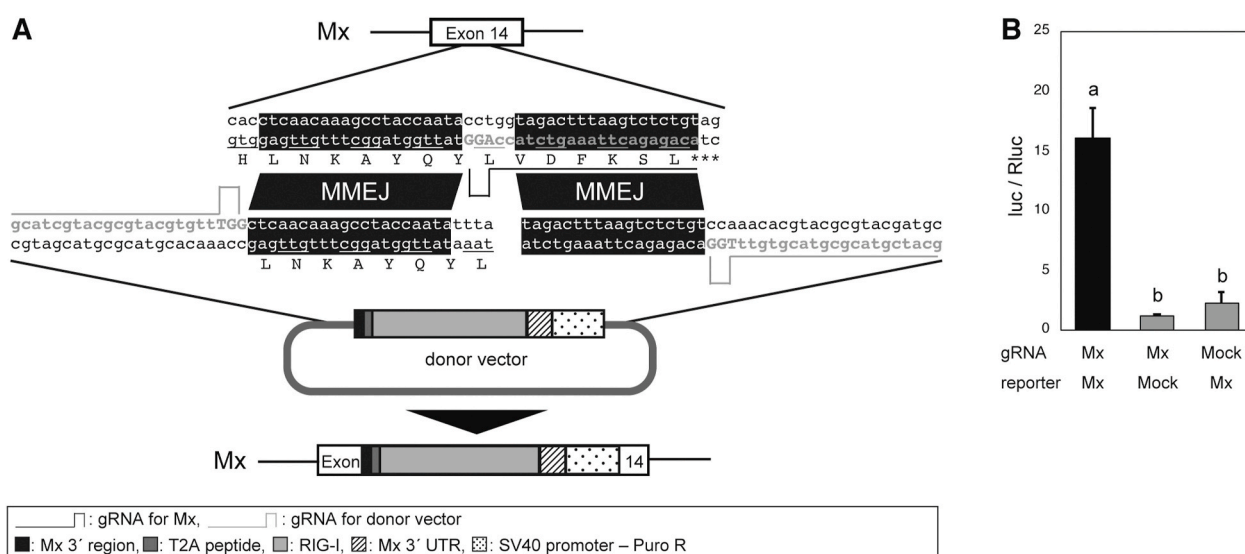
A schematic image of the knock-in using the PITCh method is shown in Fig. 1A. A donor vector containing microhomologies, 3' region of the *Mx* gene, T2A sequence, duck *RIG-I*, 3' UTR of the *Mx* gene, and a puromycin resistance gene was constructed using the Gibson assembly method [15,16] and sequenced to verify the nucleotide sequence (Fig. S2). A schematic diagram of the vector construction is shown in Fig. S1. In this study, two types of gRNA vectors targeting the donor vector or *Mx* was constructed, and the nucleotide sequences of the gRNA vectors were confirmed (data not shown). Cleavage activity of the gRNA vector targeting *Mx* was analyzed by SSA assay, and the activity was observed (Fig. 1B).

#### 3.2. Establishment of the *RIG-I* knock-in DF-1 cell line

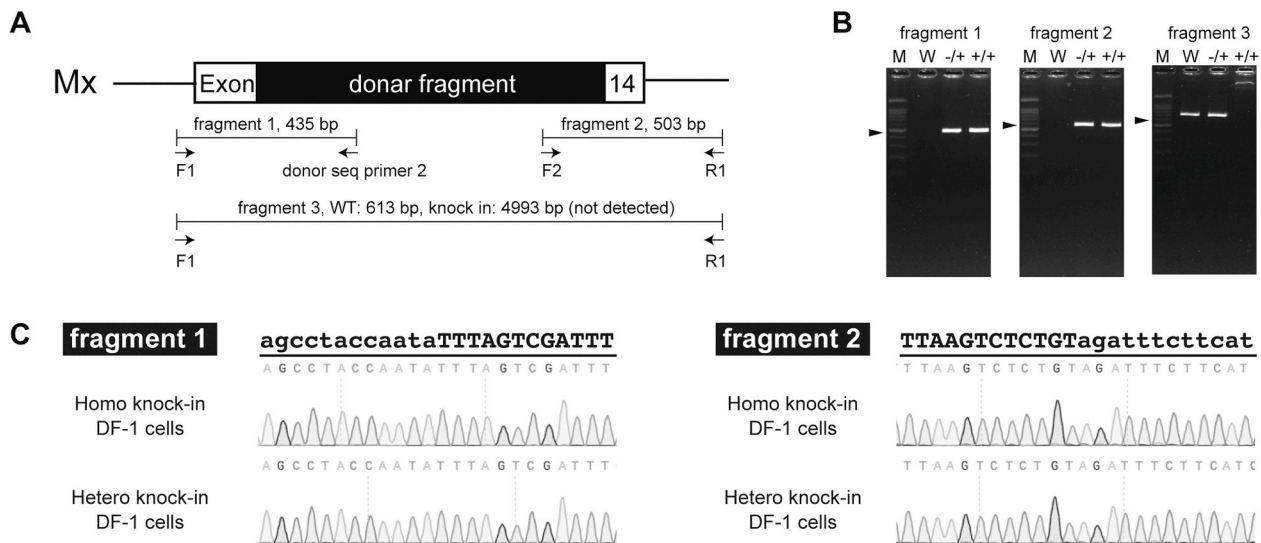
The donor vector and two different gRNA vectors were transfected into the DF-1 cells. After puromycin selection, the DF-1 cells were cloned. For screening, the PCR was performed using three pairs of primers: primer F1– donor seq primer 2, F2–R1, and F1– R1 (Table S1). A schematic diagram of the positions of these primers in the insertion sites is shown in Fig. 2A. In this screening, both the homo- and hetero-knock-in DF-1 cells were obtained (Fig. 2B). The nucleotide sequence of fragments 1 and 2 obtained by PCR in the knock-in DF-1 cells revealed that the donor fragment was correctly inserted into both the homo- and hetero-knock-in DF-1 cells (Fig. 2C).

#### 3.3. Expression analysis of *RIG-I* under stimulation of chicken IFN- $\beta$ and poly(I:C)

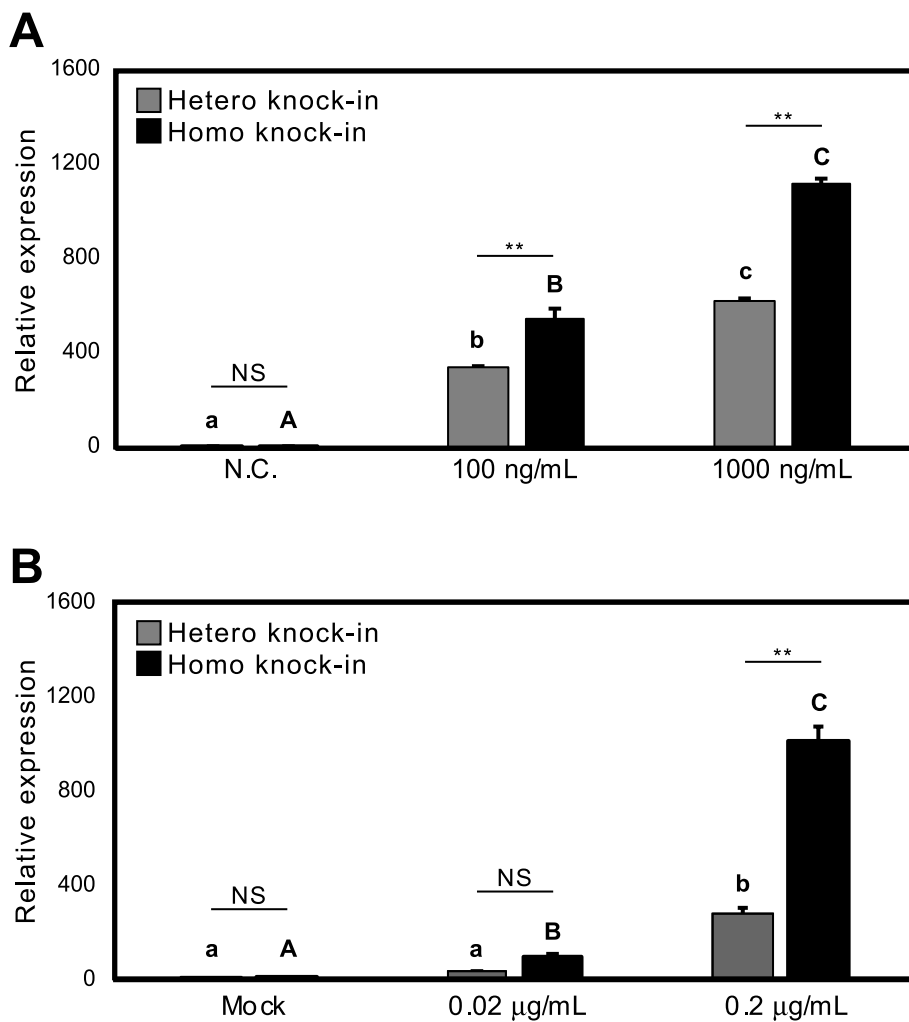
In a previous study, it was revealed that the ISRE sequence, which is activated by IFN stimulation, is located in the promoter region of the chicken *Mx* genome [8]. Therefore, we stimulated both the homo- and hetero-knock-in DF-1 cells by recombinant chicken IFN- $\beta$  to evaluate the *RIG-I* expression patterns. *RIG-I* expression was regulated by IFN- $\beta$  stimulation in a dose-dependent manner (Fig. 3A). Moreover, to compare the expression levels of *RIG-I* in homo-knock-in DF-1 cells with that in the hetero-knock-in DF-1 cells, the levels of homo-knock-in DF-1



**Fig. 1.** A strategy for the establishment of *RIG-I* knock-in DF-1 cells. (A) A schematic illustration of the knock-in of *RIG-I* using the precise integration into target chromosome method. Open boxes indicate microhomologies. The stop codon is marked with three asterisks. (B) Single-strand annealing (SSA) assay using the *Mx* gRNA vector and SSA vector including the *Mx* gRNA targeted site. Error bars indicate SD of the mean ( $n = 3$  wells/condition), and significance was evaluated by Tukey's test. Different alphabets represent significant difference ( $P < 0.01$ ).



**Fig. 2.** Genotyping of *RIG-I* knock-in DF-1 cells. (A) A schematic image for the screening of *RIG-I* knock-in DF-1 cells. *Mx* exon 14 (open box) and inserted donor fragment (closed box) are shown. Primers used in this analysis are designated by arrows. (B) Gel electrophoresis of the PCR fragments. The predicted size of each fragment is indicated by arrowheads. M, 100 bp marker; W, wild type DF-1; -/+, hetero knock-in DF-1; and +/+, homo knock-in DF-1. (C) The results of the sequencing analysis. Small and capital letters indicate nucleotide sequence of exon 14 in the *Mx* gene and donor fragment, respectively.

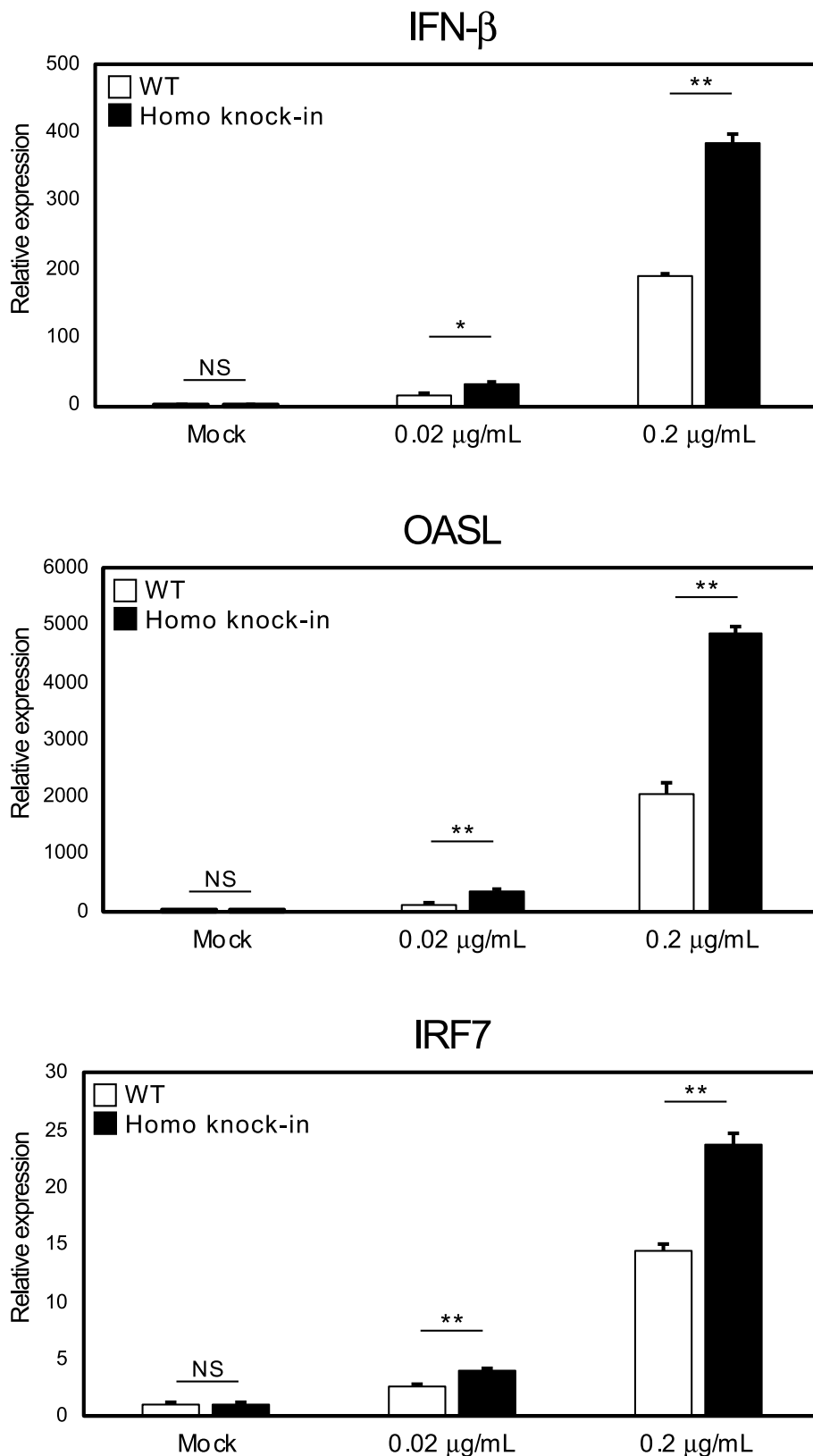


**Fig. 3.** Expression analyses of *RIG-I* by qPCR. (A) The expression analysis under recombinant chicken IFN- $\beta$  stimulation. (B) The expression analysis under poly(I:C) stimulation. In the mock condition, the lipofection reagent was administered without poly(I:C). The  $2^{-\Delta\Delta Ct}$  method was used for the calculation of relative expression scores, which were normalized by levels of  $\beta$ -actin. The expression levels were analyzed by technical triplication (n = 3). Error bars indicate SD of the mean. Significance was evaluated by two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. The different letters represent significant differences between the same genotypes (P < 0.05). Asterisks represent significant differences between hetero- and homo-knocked-in DF-1 cells (\*\*P < 0.01). NS means no significant differences. The significant differences were observed in an independent experiment in IFN- $\beta$  and poly(I:C) stimulation, respectively.

cells were approximately 2-folds higher than that in DF-1 cells under IFN- $\beta$  stimulation. This result demonstrated that the genotypes of each cloned cell corresponded to the phenotypes.

Poly(I:C) is a synthetic dsRNA that is generally used to mimic a virus. In this study, poly(I:C), with an average size of 0.2–1 kb, was used as a

ligand of RIG-I. The expression patterns of RIG-I were analyzed by qPCR in both homo- and hetero-knock-in DF-1 cells. RIG-I expression was highly upregulated by poly(I:C) stimulation in a dose-dependent manner (Fig. 3B). Therefore, a ligand stimulation-dependent expression pattern for the inserted RIG-I was observed.



**Fig. 4.** Expression analyses of innate immune response-related genes under poly(I:C) stimulation. In the mock condition, the lipofection reagent was administered without poly(I:C). The  $2^{-\Delta\Delta C_t}$  method was used for the calculation of relative expression scores, which were normalized by levels of  $\beta$ -actin. The expression levels were analyzed by technical triplicates ( $n = 3$ ). Error bars indicate SD of the mean. Significance was evaluated between WT and homo-knock in the DF-1 cells under the same conditions by Student's  $t$ -test. (\* $P < 0.05$ , \*\* $P < 0.01$ ). NS, no significant differences. Representative data from three individual experiments are shown.

To confirm the co-transcription of *RIG-I* with *Mx* via the T2A peptide sequence, RT-PCR was performed. A schematic diagram of this RT-PCR is shown in Fig. S3A. The forward primer (RT-F) and the reverse primer (RT-R) targeted the *Mx* and the *RIG-I* gene, respectively. The fusion cDNA was detected in the homo-knock in DF-1 cells under poly(I:C) stimulation (Fig. S3B).

### 3.4. Expression analyses of innate immune response-related genes under poly(I:C) stimulation

The expression levels of some immune response-related genes, *IFN- $\beta$* , 2'-5'-oligoadenylate synthase-like protein (*OASL*), and interferon regulatory factor 7 (*IRF7*), in homo-knock-in DF-1 cells were compared to those in WT DF-1 cells under poly(I:C) stimulation. The expression levels were upregulated by poly(I:C) stimulation in a dose-dependent manner in both homo-knock-in and WT DF-1 cells (Fig. 4). Compared to the expression levels of these genes in WT DF-1 cells, those in homo-knock-in DF-1 cells were significantly higher under poly(I:C) stimulation at both 0.02 and 0.2  $\mu\text{g}/\text{mL}$ . Interestingly, there were no differences in the expression levels of these genes between mock-transfected homo-knock-in and WT DF-1 cells. Therefore, *RIG-I* expression depends on *Mx* promoter activity and is functional for upregulation of the innate immune response genes by ligand stimulation.

## 4. Discussion

In the present study, *RIG-I* was inserted into the 3' region of the *Mx* gene using the PITCh method (Fig. 1). Homo- and hetero-knock-in DF-1 cells were then obtained (Fig. 2). The knock-in DF-1 cells expressed *RIG-I* upon recombinant chicken *IFN- $\beta$*  stimulation, and the expression levels were dependent on the genotypes (Fig. 3A). The ISRE motif of the *Mx* promoter is closely related to *IFN* response [8]. Therefore, it has been suggested that the upregulation of the inserted *RIG-I* under recombinant chicken *IFN- $\beta$*  stimulation was dependent on the ISRE motif in the *Mx* promoter. In ducks, the ISRE motif was also contained in the *RIG-I* promoter and affected the promoter activity [7]. Hence, in terms of *IFN* response, the expression pattern of *RIG-I* in knock-in DF-1 cells corresponds to that in duck cells.

Transfection of poly(I:C) also induced the upregulation of *RIG-I* and innate immune response-related genes, *IFN- $\beta$* , *OASL*, and *IRF7*, in the knock-in DF-1 cells (Figs. 3B and 4). In the duck RLR family signaling pathway, activated *RIG-I* interacts with mitochondrial antiviral-signaling protein (MAVS). Then, the MAVS proteins lead to both activation and nuclear translocation of *IRF7*, a transcription factor related to interferon expression. Subsequently, the expression of type-I *IFN* upregulates RLRs and ISGs, including *OASL* [5]. The upregulation of these genes due to poly(I:C) stimulation and *IFN- $\beta$*  response of *Mx* promoter in the knock-in DF-1 cells suggested that the inserted *RIG-I* induces a positive feedback as shown in the RLR family signaling pathway in duck cells. The expression levels of *IFN- $\beta$*  in homo-knock-in DF-1 cells were only 2-folds higher than that in WT DF-1 cells. However, overexpression of *RIG-I* in DF-1 cells also induced a 2-fold increase in *IFN- $\beta$*  expression compared to WT DF-1 cells under poly(I:C) stimulation, despite the acquisition of resistance for HPAIV [6]. Therefore, the *IFN* inducibility of *RIG-I* expressed under the *Mx* promoter corresponded to *RIG-I*-overexpressing DF-1 cells.

Here, we showed that the regulation of *RIG-I* expression under *Mx* promoter activity can enhance the innate immune response without overexpression. In this study, we attempted to address problems due to overexpression and random integration of duck *RIG-I* in chickens. Our results showed the effectiveness of this strategy to induce the innate immune response dependent expression of *RIG-I* in chicken cells without host gene disruption.

In the future, the effects of AIV resistance on the expression control of *RIG-I* under the *Mx* promoter should be analyzed both *in vitro* and *in vivo*. In ducks, *RIG-I* and *Mx* have similar expression patterns after a AIV

infection; white pekin ducks infected with the H5N1 strains induces *RIG-I*, and *Mx* at 1 day post-infection in both the lung and spleen and modest upregulation of these genes persisted to 3 days post-infection [17]. On the other hands, in chickens, *Mx* was also upregulated by infection of HPAIV strains in the lung, spleen, and brain at 1 day post-infection [18]. Therefore, the chicken *Mx* promoter has potential as a controller of *RIG-I* during AIV infection in chickens. Our findings indicate the prospect of establishing transgenic chickens that expressed *RIG-I* stably and dependent on immune response.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2021.101084>.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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