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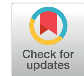
gga-miR-142-5p attenuates IRF7 signaling and promotes replication of IBDV by directly targeting the chMDA5′s 3′ untranslated region



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ABSTRACT

Chicken melanoma differentiation-associated gene 5 (*chMDA5*) is a key pattern recognition receptor (PRR) that recognizes RNA viral infections and initiates an antiviral innate immune response in chickens. MicroRNAs (miRNAs) are involved in the regulation of *chMDA5* to sense RNA virus infection, but how it exerts antiviral activity against infectious bursal disease virus (IBDV) infection and regulates *chMDA5* in chicken cells is unclear. Thus, we measured the expression of chMDA5 in IBDV-infected DT40 cells and found it significantly increased. Overexpression of chMDA5 activated the IFN-β and Mx promoters via IRF7-dependent pathways and inhibited replication of IBDV in DT40 cells. The opposite effect occurred after chMDA5 knockdown using siRNA. Also, gga-miR-142-5p regulated chMDA5 according to bioinformatic analysis and data from a dual-luciferase reporter system. Overexpression of gga-miR-142-5p reduced the expression of the chMDA5 protein, promoting IBDV replication, and decreased the activity of the IFN-β and Mx promoters via an IRF7-dependent pathway; however, it had no effect on the NF-κB-dependent pathway in DT40 cells. Thus, gga-miR-142-5p is a negative regulator of chMDA5 and promotes IBDV replication in DT40 cells through an IRF7-dependent pathway.

1. Introduction

Infectious bursal disease virus (IBDV), of the genus *Avibirnavirus* within the family *Birnaviridae*, is the causative agent of infectious bursal disease (IBD) (Wang et al., 2007). IBDV mainly invades the bursa of Fabricius of young birds, causing death or immunosuppression in surviving birds and increased their susceptibility to other pathogenic infections. Vaccination is critical for preventing and controlling avian IBDV infection, but the IBDV genome is easily mutated. Highly virulent variant natural recombinant strains of IBDV often emerge and cause vaccination failure, creating a persistent challenge to prevention and control measures of IBDV (Ingrao et al., 2013; Gallardo et al., 2014; He et al., 2014).

Innate immunity is required for acquired immunity against viral infection via recognition of pathogen-associated molecular patterns (PAMPs) of microorganisms using pattern recognition receptors (PRRs). The activation of PRRs by PAMP triggers the expression of type I interferons, inflammatory cytokines, and chemokines, which plays a critical role in host to defense upon virus infection (Akira et al., 2006; Kawai and Akira, 2007). Retinoic acid-inducible gene I (RIG-I) is one of the key innate immune PRRs. The retinoic acid-inducible gene I (RIG-I) receptor (RLR) family contains three members, includes retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetic and physiology 2 (LGP2), which are chiefly localized to the cytoplasm. PAMPs of microbes, including viral genomic DNA, single-strand RNA (ssRNA), double-strand RNA (dsRNA), RNA-containing 5′-triphosphate groups and viral proteins, can be recognized by RIG-I (Takeuchi and Akira, 2010; Chen et al., 2013). Human-derived RIG-I and MDA5, two key PRRs that activate host interferons, are evolutionarily conserved in their molecular structure and have roles that are relatively consistent. They recognize intracellular nucleic acids (including viral genomes and the intermediates of nucleic acids produced during viral replication) (Matsumiya and Stafforini, 2010; Karpala et al., 2011). Chicken cells lack RIG-I, so MDA5 compensates for this loss of function (Matsumiya and Stafforini, 2010). Although The mechanism of RNA viruses recognized by MDA5 in mammals are well understood, how chicken MDA5 identifies microbe PAMPs and initiates signal transduction pathways of the innate immune system responses has not been well understood.

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Increasing evidences indicate that miRNAs have important functions to regulate PRRs expression in response to virus infections. MiRNA can target innate immune signaling pathway molecules, such as PRRs, joint proteins, kinases, transcription factors, and inflammatory cytokines (Zhou et al., 2011; O'neill et al., 2011). However, how miRNAs regulate chicken chMDA5 during antiviral innate immune signal transduction was under explored. Therefore, we studied IBDV infection-mediated stimulation of chMDA5 expression and noted the initiation of downstream signaling pathways that inhibit IBDV infection. Moreover, we investigated regulatory mechanisms of chMDA5 at the miRNA level during an antiviral innate immune response in chicken cells.

2. Materials and methods

2.1. Cell culture and viral infection

Pre-B-lymphocyte DT40 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 5% chicken serum, 10% fetal bovine serum, 50 μM β-mercapto-ethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. An IBDV B87 DT40 cell adapted strain (moderately virulent live vaccine strain, which was produced in DT40 cells) was used in this study.

For IBDV infection, DT40 cells (90% confluency) were infected with 1000 $TCID_{50}$ (the 50% tissue culture infectious dose) IBDV B87 DT40 cell adapted strain. After 1 h of incubation, the cells were washed twice with DMEM supplemented with 2% fetal bovine serum and then re-fed with DMEM supplemented with 2% fetal bovine serum. 24 h after inoculation with the virus, both the supernatant and the cells were collected. The virus titers in the supernatant were analyzed using $TCID_{50}$, and the total RNA was obtained from the cells using Trizol reagent (Takara) in accordance with the manufacturer's instructions.

2.2. Plasmid construction

Total RNA was extracted from IBDV-infected-DT-40 cells using Trizol reagent (Takara). Reverse transcription was performed by 5 × All-In-One RT MasterMix (with AccuRT Genomic DNA Removal Kit) purchased from ABM (Vancouver, Canada) according to the manufacturer's instructions. The open reading frame of chMDA5 gene was amplified from the cDNA of IBDV-infected-DT-40 cells by PCR. The PCR products were purified and subcloned into the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA, USA) to generate the recombinant vector, pcDNA3.1-Flag + chMDA5. The pcDNA3.1-Flag + chMDA5 was identified by double restriction enzymes (*Nhe*I and *Xba*I) digestion and sequence analysis.

Chicken IFN-β and chicken Mx promoters were amplified from chicken genomic DNA using PCR with the following primers: IFN-β promoter (F) and IFN-β promoter (R), Mx promoter (F) and Mx promoter (R), respectively (Primer sequences can be made available upon request). PCR products were purified and subcloned into pGL3-basic (Promega), generating the recombinant vector, pGL3-Basic + IFN-β and pGL3-Basic + Mx promoters. Recombinant vectors were confirmed using double restriction enzymes (*Kpn*I and *Hin*dIII) digestion and sequence analysis. DNA sequences for assaying chicken NF-κB (chNF-κB) and chicken IRF7 (chIRF7) binding positive regulatory domain activity were synthesized, chNF-κB sequeces, which contained four copies of the chNF-κB binding positive regulatory domains motif (GGGAATTCTC) of the chicken IFN-β promoter (Wei et al., 2014); chIRF7 sequeces, which contained four copies of the chNF-chIRF7 binding positive regulatory domain motif (TTCACTTTCAATA) of the chicken IFN-β promoter (Wei et al., 2014). chNF-κB and chIRF7 were subcloned into the pGL3-control (Promega) to yield the recombinant vector, pGL3-Luc-NF-κB and pGL3-Luc-IRF7, respectively. The recombinant vectors were both confirmed by double restriction enzymes (KpnI and HindIII) digestion and sequence analysis, respectively.

The chMDA5 3′-UTR were amplified from chicken genomic DNA by PCR. The PCR products were purified and subcloned into the pGL3-control (Promega), resulting in the generation of the recombinant vector, pGL3-chMDA5 3′UTR. The recombinant vectors were confirmed by double restriction enzymes (*Kpn*I and *Xho*I) digestion and sequence analysis. All primers and DNA oligonucleotides were synthesized by TsingKe, China (Primer sequences can be made available upon request). PCR products were purified using an EasyPure Quick Gel Extraction Kit (TransGen Biotech, China).

2.3. RNA interference

Two siRNAs were designed using the online tool for siRNA design (http://rnaidesigner.lifetechnologies.com/rnaiexpress/) based on the conservative region of chMDA5. Sense and antisense strand DNA oligonucleotide sequences for siRNAs and control siRNA sequences can be made available upon request. Synthesizd single-strand DNA oligonucleotides were diluted with nuclease-free water, and paired and double-strand DNA templates for transcription of siRNAs were obtained. The reaction system included 10 μL sense DNA oligonucleotides (100 pmol/μL), 10 μL antisense DNA oligonucleotides (100 pmol/μL), 20 μL 5 × Oligo Annealing Buffer, 60 μL nuclease-free water. The reaction system was heated to 95 °C for 5 min and annealed at room temperature for 30 min. Transcription of siRNA was performed according to the T7 RiboMAX Express RNAi System kit purchased from Promega (Madison, WI, USA). siRNA duplexes were transfected into DT40 cells (final concentration 10 nM) using Lipofectamine RNAiMAX supplied by Invitrogen (Carlsbad, CA, USA), according to the manufacturer's instructions.

2.4. Bioinformatic prediction of miRNAs targeting chMDA5

miRDB (http://mirdb.org/miRDB/) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) were used to predict miRNAs targeting chMDA5. gga-miR-142-5p mimics were modified oligonucleotides for gga-miR-142-5p (CCCAUAAAGUA GAAAGCACUAC) and gga-miR-142-5p inhibitors were modified anti-gga-miR-142-5p oligonucleotides. gga-miR-142-5p mimics and gga-miR-142-5p inhibitors, used for overexpression and inhibition of gga-miR-142-5p activity respectively in DT40 cells, were synthesized by Guangzhou RiboBio (China). DT40 cells were transfected with miRNAs (100 nM) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Negative control mimics or inhibitors (RiboBio) were transfected as matched controls.

2.5. Luciferase assay

DT40 cells were cultured in six-well plates to 50–70% confluency and cotransfected with pGL3-chMDA5 3′UTR, pRL-CMV, and gga-miR-142-5p mimics or miRNA mimic control. Then, 24 h later, luciferase activity was measured using a reporter gene assay kit. pcDNA3.1-Flag + chMDA5 or the empty plasmid pcDNA3.1(+) was cotransfected with pGL3-Basic + IFN-β or pGL3-Basic + Mx promoter and pRL-CMV, according to Lipofectamine 2000 kit instructions. Then, 24 h later, luciferase activity was measured according to the instructions of Dual Luciferase Reporter Gene Assay Kit purchased from Promega (Madison, WI, USA). si-chMDA5 or siRNA-Control was cotransfected with pGL3-Basic + IFN-β or pGL3-Basic + Mx promoter and pRL-CMV, and then infected with 1000 $TCID_{50}$ IBDV B87 24 h post transfection. Luciferase activity was measured according to the instructions of Dual Luciferase Reporter Gene Assay Kit purchased from Promega (Madison, WI, USA).

2.6. Western blot

DT40 cells were infected with IBDV B87 and collected at different time points (0, 2, 4, 12, and 24 h). chMDA5 mRNA was analzyed with qRT-PCR and expression of chMDA5 protein was assayed using Western

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