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Overexpression of microRNA gga-miR-21 in chicken fibroblasts suppresses replication of infectious bursal disease virus through inhibiting VP1 translation

Yong-shan Wang [a,*], Wei Ouyang [a], Qun-xing Pan [a], Xiao-li Wang [a], Xing-xia Xia [a], Zhen-wei Bi [a], Yong-qiang Wang [b], Xiao-mei Wang [b,*]

[a] Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture, Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China
[b] State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, China

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ABSTRACT

Previously we have identified a series of cellular miRNA molecules up- or down-regulated in infectious bursal disease virus (IBDV) infected chicken embryo fibroblasts and Bursa of Fabricius with gene microarray analysis. Here we studied in detail a relatively well studied miRNA, gga-miR-21, for better understanding miRNAs involvement in IBDV-host interactions. Chicken pri-gga-miRNA-21 and a control miRNA *Caenorhabditis elegans* pri-cel-lin-4 gene were cloned into a lentiviral vector, respectively. The resulting recombinant lentiviruses were used to infect chicken fibroblast cell line DF-1, and two stable cell lines, DF-miR-21 (overexpressing gga-miR-21) and DF-lin-4 (overexpressing cel-lin-4), were selected. Replication of IBDV in DF-miR-21, DF-lin-4 and DF-1 cells were compared and molecular mechanism of IBDV replication alteration was explored using bioinformatics, reporter gene system, qRT-PCR and Western blot analysis. IBDV replication was markedly lower in DF-miR-21 than in DF-lin-4 or DF-1 cells. A gga-miR-21 target sequence was identified within IBDV VP1 gene (1713–1734 bp). Fusion of a 520nt long partial IBDV VP1 gene containing the target with a luciferase gene resulted in significantly lower transient luciferase activity in DF-miR-21 cells as compared to that in DF-lin-4 or DF-1 cell. Following IBDV infection of the cell lines, VP1 protein level in DF-miR-21 cells was dramatically lower than that in DF-lin-4 or DF-1 cells but VP1 mRNA level was not different. The finding indicated that gga-miR-21 could suppress IBDV replication through down regulating IBDV VP1 expression at translational level.

1. Introduction

Infectious bursal disease virus (IBDV) infects chicken lymphoid tissues, especially the central immune organ – bursal, causing deaths or immunosuppression, leading to weakened immunity and vaccination failures. IBDV is hard to eradicate due to its biological stability, high variability, and carriage and transmission by a variety of bird species (Wang et al., 2007; Wang et al., 2008). Currently, IBD is still among the major infectious diseases of the poultry industry (Dolz et al., 2005; Wang et al., 2012).

IBDV is a double stranded RNA virus with two genome segments (A and B) encoding total five viral proteins VP1 to VP5. VP1 is encoded by segment B and is a RNA-dependent RNA polymerase. VP2 to VP5 are encoded by segment A in two partially overlapping open reading frames (ORF). The larger ORF encodes a poly-protein which is processed into VP2, VP4 and VP3. VP2 is both the major structural protein and the major protective antigen of the virus (Fernández-Arias et al., 1997). VP3 and viral RNA form helical nucleoprotein complex which is located inside the virion (Tacken et al., 2002). VP4 is a viral protease (King et al., 2012). The smaller ORF encodes VP5, which is a non-structural protein related to virus virulence and efficiency of virus replication (Lombardo et al., 2000).

A large number of studies have been carried out on IBDV etiology, ecology and epidemiology (Müller et al., 2003). However, studies on IBDV pathogenesis have remained at the stage of describing clinical symptoms and pathological changes and detecting and analyzing cellular and humoral immune responses, and there is a lack of in-depth exploration of molecular mechanisms of interactions between IBDV and its target cells.

Gene regulation is complicated and occurs on multiple levels by different means. MicroRNA (miRNA) is a type of small, non-coding RNA of 21 ~25nt in length and widespread in eukaryotes (Guarnieri and Dileone, 2008). Cellular endogenous miRNA can serve as a

* Corresponding authors. Tel./fax: +86 25 84391173 (Y.-S. Wang).
E-mail addresses: wangys63@126.com, wangys1963@gmail.com (Y.-s. Wang), xmw@hvri.ac.cn (X.-m. Wang).

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type of guiding molecules through base pairing with their target mRNA, thereby leading to post-transcriptional splicing or translation inhibition of the target mRNA, which play key roles in different regulatory pathways including various physiological and pathological processes (Lim et al., 2003). Increasing evidences suggest that cellular miRNAs play key regulatory roles during viral infections. Alterations in cellular miRNA expression during virus infection are part of host–virus interactions and play a key role in the regulation of viral replication (Song et al., 2010; Umbach and Cullen, 2009). Profiling analyses have been employed to detect miRNA changes in tissues infected by viruses, which could help to reveal molecular pathways regulating viral pathogenesis. However, expression profiles of cellular miRNAs in hosts infected by IBDV have not been reported so far.

In order to analyze the impact of cellular miRNA on IBDV replication, we have previously used microarray technology to analyze miRNA expression changes in chick embryo fibroblasts (CEF) and Bursa of Fabricius of SPF chickens after their infection by different IBDV strains and found IBDV infection caused significant changes in expression of multiple cellular miRNAs (Ouyang et al., 2012).

In this study, to further previous results of differential miRNA expression in IBDV infected chicken cells, we chose to study in detail one up-regulated miRNA, gga-miR-21. We established a chicken fibroblast cell line overexpressing gga-miR-21 and studied IBDV replication in these cells and explored related mechanisms.

2. Materials and methods

2.1. Virus, cells and main reagents

IBDV B87 CEF adapted strain (a moderate-virulent live vaccine strain), 293FT cells, DF-1 cells (chicken embryo fibroblast cell line) and *Caenorhabditis elegans* were kept in this laboratory. The lentiviral expression system (lentiviral vector pLenti6-EGFP and packaging vectors pLP1, pLP2 and pLP/VSVG) was a gift from professor Honglin Liu of Nanjing Agriculture University. Molecular cloning vectors, enzymes, reagents and kits were from TaKaRa (Japan). Lipofectamine 2000 transfection reagent and blasticidin were from Invitrogen (USA). All primers were synthesized by Invitrogen. Hexadimethine bromide (Polybrene) was from Sigma. Dual luciferase reporter vectors (pRL-CMV, pGL3-control) and assay kits were from Promega.

2.2. IBDV $TCID_{50}$ assay

$TCID_{50}$ for IBDV is defined as the dilution of virus that would produce a cytopathic effect (CPE) in 50% of the infected DF-1 cells. DF-1 Cells were plated in 96-well plates at 3×10^4 cells/well density 24 h prior to the assay to achieve 75–90% confluency. Ten-fold serial dilutions from 1:10 to $1:10^8$ were prepared for each sample, and from each dilution 100 μL/well of virus suspension was added to the cells in quadruplicates. Wells with CPE were scored as positive for virus growth. Virus-infected cultures were recovered and titrated by $TCID_{50}$ assay.

2.3. Generation of gga-miR-21, cel-lin-4 overexpressing cell lines with a lentiviral expression system

Sequence of mature gga-miR-21, obtained from miRBase (http://www.mirbase.org/), was used to search chicken genome database (http://www.ensembl.org/index.html) to yield pri-gga-miR-21 sequence, which was ~520nt in length, containing the 22nt mature gga-miR-21 and flanking sequences of 250nt on each side. Primers for pri-gga-miR-21 amplification were: pri-gga-miR-21(F): 5′-CGGAATTCGATTAGCCTAGGGGGATT-3′ (EcoRI) and pri-gga-miR-21(R): 5′-GCGTCGACGAAGTTATACACGAAG CATAC-3′ (Sal I).

The pri-gga-miR-21 sequence was amplified from genomic DNA of liver tissues of a 60-day Leghorn chicken and was TA-cloned into vector pMD18-T, excised by EcoR I and Sal I and subcloned into pLenti6-EGFP (a modified version of pLenti6/V5-GW/lacZ in which the miRNA is expressed from the GFP reporter), yielding pL-miR-21. Then pL-miR-21 and three packaging vectors pLP1, pLP2 and pLP/VSVG were co-transfected into 293FT cells with Lipofectamine 2000. At 12 h post transfection, medium was replaced with complete DMEM medium containing 10% calf serum. At 72 h, virus-containing supernatants were harvested, spun at 4000 r/min for 10 min to remove cell debris and solutions with the recombinant lentivirus containing pri-gga-miR-21 particles $V_{L+miR\text{-}21}$ were obtained.

The $V_{L+miR\text{-}21}$ particles were used to infect DF-1 cells where the medium contained polybrene at final concentration of 8 μg/mL for observation of GFP expression under fluorescence microscope. At 24 h post-infection, culture medium was replaced with fresh DMEM containing blasticidin (8 μg/ml) which was refreshed every 3–4 days until all surviving cells were GFP positive and blasticidin-resistant colonies were obtained. Individual colonies were obtained by serial dilution and maintained in DMEM containing 4 μg/ml blasticidin and the resulting cell line was named DF-miR-21.

Expression of gga-miR-21 in DF-miR-21 was determined with SYBR Green I quantitative RT-PCR (qRT-PCR) with chicken 5S rRNA as the internal reference. The reverse primers for gga-miR-21 and 5S rRNA were provided by SYBR PrimeScript miRNA RT-PCR kit, and the forward primers for gga-miR-21 and 5S rRNA were: gga-miR-21(F):5′-GCGTAGCTTATCAGACTGATGTTGA A-3′ and 5S rRNA(F): 5′-GTACTTGGATGGGAGACTGCCT-3′. The calculation method was $2^{-\triangle\triangle Ct}$ (Livak and Schmittgen, 2001).

For a DF-1 derived cell line infected with a recombinant lentivirus over-expressing a "scramble" miRNA to serve as a negative control, the miRNA cel-lin-4 of *C. elegans* was selected and parallel preparations were made. One pri-cel-lin-4 sequence was yielded from *C. elegans* genome from the sequence of mature cel-lin-4 based on miRBase (http://www.mirbase.org/). Primers for pri-cel-lin-4 were: pri-cel-lin-4 (F): 5′-CGGAATTCCTTTCGATCCTCCTCCTTC AG-3′(EcoRI); pri-cel-lin-4(R): 5′-CGGGATCCATATTCGAATAGTAAA AATGT-3′(BamHI).

A recombinant lentivirus containing pri-cel-lin-4 was prepared and used to infect DF-1 cells to yield the cell line termed DF-lin-4, in which the overexpression of cel-lin-4 miRNA was confirmed by qRT-PCR.

Growth curves of the DF-miR-21, DF-lin-4 and DF-1 cells were measured as cells were grown in 24-well plates with plating density of 2×10^4/well. The wells were in triplicates and cell growth were quantified daily. For assessing cell cycling, cells in logarithmic growth phase were grown in on glass slides in culture dishes, and at each 24 h time point over 7 days one slide was taken and fixed with 95% ethanol and treated with Giemsas staining. Dividing and non-dividing cells were counted separately under high power microscope. The experiments were repeated three times.

Stability of DF-miR-21 and DF-lin-4 cells was accessed by continuous culture for 15 passages and identification every 5 passages. Expression of GFP was observed under fluorescence microscope, and expression of gga-miR-21 or cel-lin-4 was detected by qRT-PCR.

2.4. IBDV replication in DF-miR-21, DF-lin-4 and DF-1 cells

Monolayers of DF-miR-21, DF-lin-4 or DF-1 cells were prepared, and when confluence reached 60%, each well was inoculated with

100TCID$_{50}$ IBDV B87 strain. Culture supernatant was collected at multiple time points within 24–96 h for IBDV titer determination.

2.5. Prediction of gga-miR-21 binding sequences in IBDV mRNAs

Gga-miR-21 target sequence in IBDV B87 genome was predicted using the RNA22 miRNA target predictor at http://cbcsrv.watson.ibm.com/rna22.html to be 1713–1734 bp of IBDV genome B segment. The identified miRNA-target sequence was confirmed using a second method, RNAHybrid, at http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html (see Fig. 3).

2.6. Assessing impact of gga-miR-21 overexpression with dual luciferase reporter system

Primers were designed for IBDV VP1 gene at about 250nt on each side from the predicted gga-miR-21 target sequence of 1713–1734 bp of IBDV genome B segment: IBDV-VP1′(F): 5′-CGGGATCCGCTCTAGTGGTGGACTCATCGTGC-3′(BamHI) and IBDV-VP1′(R): 5′-CGGGATCCACCTACCAACCTCAACGCCTCATAC-3′(BamHI). A ~520 bp fragment of IBDV VP1 segment was then amplified by RT-PCR from the total RNA extracted from IBDV-infected cells, digested with BamH I, and directionally cloned into the 3′UTR of the firefly luciferase gene in the pGL3-control vector (Promega) to yield pGL3-VP1′. Similarly, a mutated fragment of the gga-miR-21 target sequence which containing a multiple point mutations in gga-miR-21 binding site (Fig. 3), was also generated and cloned into the 3′UTR of the firefly luciferase gene in the pGL3-control vector to construct the plasmid pGL3-VP1′ mutant.

DF-miR-21, DF-lin-4 and DF-1 cells were seeded in 6-well plates, respectively, and were co-transfected with 3 μg of pGL3-VP1′ or pGL3-VP1′ mutant (firefly luciferase reporter) and 1 μg of pRL-CMV (Renilla luciferase reporter) using Lipofectamine 2000. The cells were harvested 48 h later after transfection and split into 96-well plates (in triplicates) for luciferase assays using a luciferase assay kit (Promega) according to the manufacturer's protocol. Firefly luciferase activity was normalized using Renilla luciferase activity.

2.7. Western blot analysis of IBDV protein expression in DF-miR-21, DF-lin-4 and DF-1 cells

DF-miR-21, DF-lin-4 and DF-1 cells were seeded in 6-well plates and infected with 200TCID$_{50}$ of IBDV B87. The plates were then incubated at 37 °C, 5% CO_2 in a humidified incubator for 1 h, at which point the infection medium was replaced with growth medium. After 72 h of incubation, the plates were freeze–thawed 3 times, and supernatants were collected. Western blot analysis was performed following standard procedures with 12% SDS–PAGE gels and nitrocellulose membranes. The primary antibody was a mouse anti-VP1 monoclonal antibody developed by this laboratory. The goat anti-mouse secondary antibody (Pierce) was conjugated with horseradish peroxidase. Proteins were detected using ECL reagents (Pierce).

2.8. Quantitative real-time PCR detection of IBDV VP1 mRNA expression

DF-miR-21, DF-lin-4 and DF-1 cells were respectively infected by IBDV B87 strain as described above in Western blot analysis. At 48 h after infection, total RNA was isolated from the cells by Trizol reagent according to the manufacturer's instructions, and 2 μg of total RNA was reverse transcribed into cDNA. Then 2 μl of reverse transcription reaction mixture was used for real-time PCR by using gene specific primers and SYBR PrimeScript RT-PCR kit (TaKaRa) according to the manufacturer's protocol. The mRNA level of housekeeping gene β-actin was measured as the internal control. The gene specific primer used were: VP1-F, 5′-CTTGCACAACCAGGGTACCTG-3′; VP1-R, 5′-CCTACCAACCTCAACGCCT-3′; β-actin-F, 5′- TTGGAGGCTCTATCCTGG-3′; and β-actin-R, 5′- TAGAAGCATTTGCGGTGG-3′. The RT-PCR product was 251 bp for VP1 and 106 bp for β-actin.

Amplification and detection were performed with LightCycler 480 II detection system (Roche). All reactions were performed in 20 μl reaction volumes and at 95 °C for 30 s, followed by 40 cycles of 94 °C for 5 s and 60 °C for 20 s. All PCR reactions were run in duplicates. Real-time PCR was monitored by the dissociation curve, agarose gel analysis and sequencing. Amounts of RNA transcripts were determined by relative quantification with standard curve. The amounts of transcripts in each sample were normalized to β-actin gene.

2.9. Statistics

Comparison was made using the Student's *t* test. In all figures, values are expressed as mean ± standard deviation (SD), and *p* value less than 0.05 was defined as statistically significant.

3. Results

3.1. Establishment of chicken fibroblast cell lines overexpressing gga-miR-21 or a control miRNA cel-lin-4

DF-1 cells were infected with recombinant lentivirus containing pri-gga-miR-21 or pri-cel-lin-4, and stable infected cells were selected with blasticidin and named DF-miR-21 or DF-lin-4 cells, respectively. As a marker of the selection system, the resulting DF-miR-21 or DF-lin-4 cells express GFP while the parent cells DF-1 do not (Fig. 1A–C). Expression of gga-miR-21 was about 60% higher than in DF-miR-21 cells than in DF-lin-4 and DF-1 cells, while the latter two had similar levels, while among the three cell lines expression of miRNA cel-lin-4 was only found in the DF-lin-4 cells (Fig. 1D). The proliferation of DF-miR-21 and DF-lin-4 cells shared similar profile as DF-1 cells. During a 7-day culture period, the numbers of cells in these three cell lines were about the same, and all the cell lines had about 24 h retention phase, 72 h logarithmic growth phase and then reached plateau phase (data not shown). The data indicated that overexpression of gga-miR-21 in DF-miR-21 or cel-lin-4 in DF-lin-4 cells did not affect proliferation characteristics of cells. In addition, for 15 continuous passages, DF-miR-21 or DF-lin-4 cells were shown to stably express GFP and gga-miR-21 or cel-lin-4, respectively (data not shown), indicating the cell lines are stably modified as designed.

3.2. IBDV replication in DF-miR-21 cells is lower than in DF-lin-4 or DF-1 cells

Next IBDV replication was compared in DF-miR-21, DF-lin-4 or DF-1 cells by measuring IBDV titer in culture supernatant at different time points through 24–96 h of infection. IBDV titer was consistently lower in DF-miR-21 cells than in DF-lin-4 or DF-1 cells, and the difference was particularly marked during 72–96 h post infection, reaching 2–3 logs (Fig. 2). This result suggests that overexpression of gga-miR-21 inhibits IBDV replication in chicken fibroblasts.

3.3. Molecular mechanism underlying gga-miR-21 overexpression and suppression of IBDV replication

To understand the molecular mechanism of IBDV replication inhibition in chicken fibroblasts overexpressing gga-miR-21, we

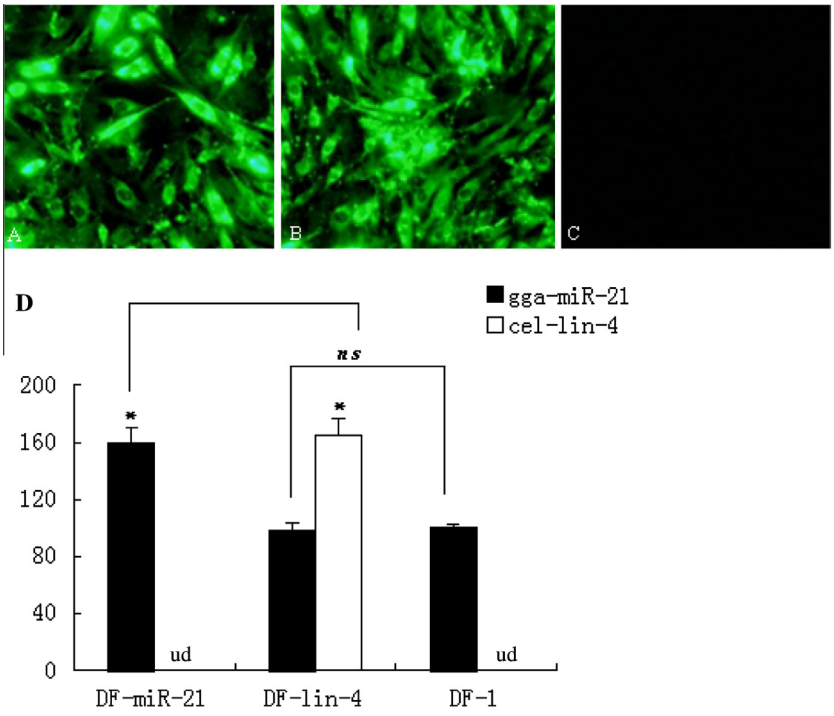

Fig. 1. Characterization of chicken fibroblast cell line DF-1 cells over expressing gga-miR-21 (DF-miR-21 cells) and cel-lin-4 (DF-lin-4 cells). DF-miR-21 and DF-lin-4 cells were obtained by infecting DF-1 cells with recombinant lentivirus containing pri-gga-miR-21 or pri-cel-lin-4 and selection with polybrene, and green fluorescent protein co-expression is a feature of DF-miR-21 (A) and DF-lin-4 (B) cells. The parental cell DF-1 (C) serves as a negative control. Fluorescence microscope (100×). (D) Detection of gga-miR-21 and cel-lin-4 expression in DF-miR-21, DF-lin-4 and DF-1 cells by qRT-PCR. $^{*}P < 0.05$.*ud* indicates undetectable.

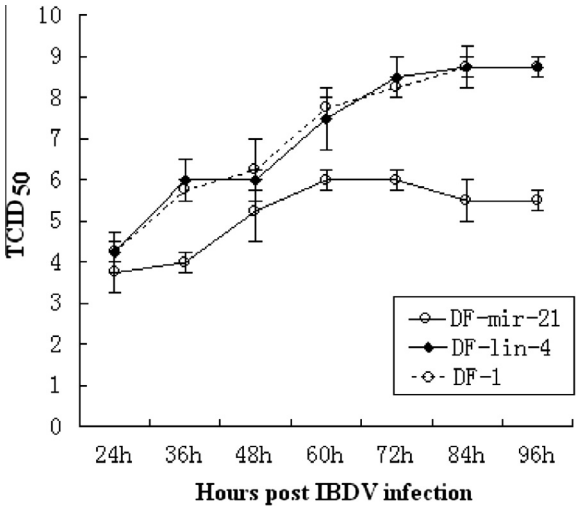

Fig. 2. Comparison of IBDV replication in DF-miR-21, DF-lin-4 and DF-1 cells. IBDV titer was lower in DF-miR-21 cells than in DF-lin-4 and DF-1 cells, suggesting overexpression of gga-miR-21 inhibits IBDV replication ($P < 0.05$).

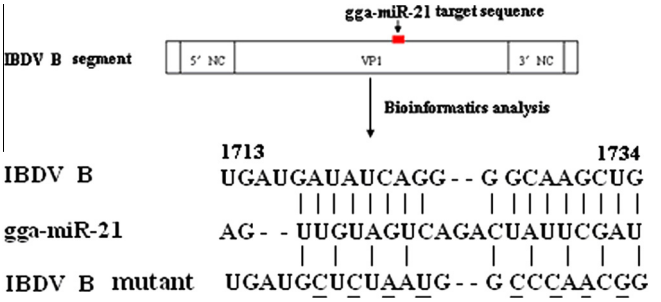

Fig. 3. Bioinformatics analysis of target sequence of gga-miR-21 in IBDV genome with software RNA22 and RNAhybrid2.2. The two methods identified the same region in IBDV genome segment B. The gga-miR-21 binding sequence was 1713–1734. The bottom row shows a mutated version of the gga-miR-21 target sequence. The mutated nucleotides are underlined.

first searched for miR-21 target sequence in IBDV genome by bioinformatics and the 1713–1734nt in IBDV genome segment B (encoding VP1) was identified (Fig. 3). It is to be noted that this 22nt is not only present in IBDV B87, but also present in at least 30 other IBDV strains in the exact sequence (22/22, 100%) according to a BLAST search.

Then we tested the miR-21 target sequence in DF-miR-21 cells by fusing the 22nt target site (or a mutated one, Fig. 3) and its flanking sequences with a luciferase gene in a dual luciferase reporter system to observe the impact of gga-miR-21 overexpression on expression of the reporter protein. Then we directly measured IBDV VP1 mRNA and protein expression in IBDV infected DF-miR-21, DF-lin-4 and DF-1 cells, respectively. These results are shown below.

3.3.1. Expression of reporter luciferase carrying miR-21 target sequence is lower in DF-miR-21 cells than in DF-lin-4 or DF-1 cells

Cells (DF-miR-21, DF-lin-4 and DF-1 cells) were co-transfected with a Renilla luciferase reporter plasmid pRL-CMV and a firefly luciferase reporter plasmid pGL3-VP1′ or pGL3-VP1′mutant or pGL3-control, respectively. Cells were harvested at 48 h post-transfection and luciferase activities were measured while the Firefly luciferase activity was normalized against Renilla luciferase activity. The firefly luciferase activity was essentially the same for pGL3-VP1′mutant or pGL3-control groups in all three cell lines. However, in DF-miR-21 cells, the firefly luciferase activity of pGL3-VP1′ was about 72% less than that of pGL3-control or

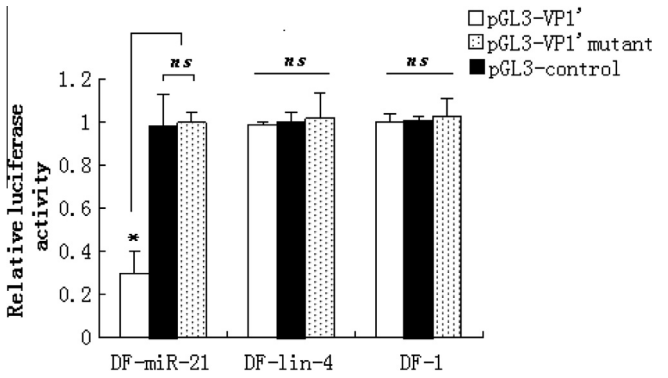

Fig. 4. Luciferase activity in DF-miR-21, DF-lin-4 or DF-1 cells transfected with a plasmid carrying a luciferase reporter gene fused with 520 bp IBDV VP1 gene fragment containing a gga-miR-21 target sequence (pGL3-VP1′) or a mutated gga-miR-21 target sequence (pGL3-VP1′mutant) or pGL3-control. *$P < 0.05$.

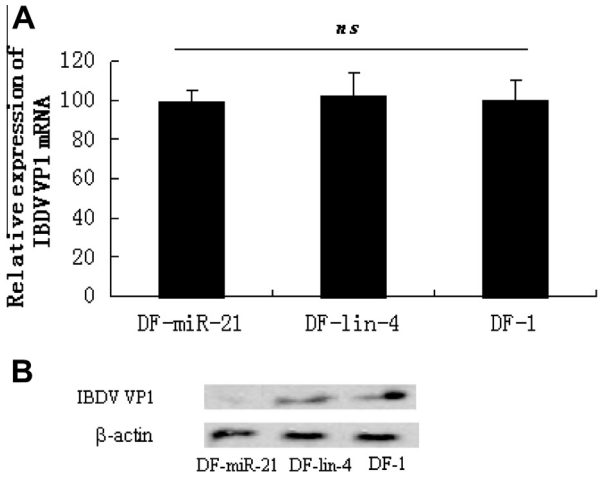

Fig. 5. Detection of IBDV VP1 mRNA and protein expression in DF-miR-21, DF-lin-4 or DF-1 cells by quantitative RT-PCR (A) or Western blotting (B), respectively. *ns* indicates no significant statistic difference ($P > 0.05$).

pGL3-VP1′mutant ($p < 0.05$); while in DF-lin-4 and DF-1 cells, firefly luciferase activity did not differ between the three plasmids (Fig. 4). The results demonstrated that expression of a reporter protein carrying gga-miR-21 target sequence is suppressed in cells overexpressing gga-miR-21.

3.3.2. IBDV VP1 protein was lower in DF-miR-21 than in DF-lin-4 or DF-1 cells but VP1 mRNA levels were comparable

DF-miR-21, DF-lin-4 and DF-1 cells were infected with IBDV B87 strain and cells were harvested at 48 h and 72 h post infection for VP1 mRNA and protein level analysis, respectively, and β-actin was detected in parallel as the internal controls (Fig. 5A and B). For VP1 mRNA, the three cell lines showed similar levels (Fig. 5A). However, VP1 protein level was markedly lower in DF-miR-21 cells than in DF-lin-4 or DF-1 cells (Fig. 5B), suggesting the inhibition of VP1 expression occurred post-transcription and at translation level.

4. Discussion

The study of how host miRNAs impact viral genes is a new approach for understanding virus–host interactions (Grassmann and Jeang, 2008). Host cell miRNAs regulate expression of viral genes, and viruses have evolved some highly complicated mechanisms to evade host immune responses. Viruses could encode miRNAs to resist cellular antiviral responses, and they could even utilize cellular miRNA pathways to assist virus replication. Hepatitis C virus appears to utilize miR-122 for its own advantage and inhibition of miR-122 binding with its target resulted in sustained suppression of HCV viremia and significant improvement in HCV-induced histopathological changes (Lanford et al., 2010). A few cellular miRNA (miR-323, miR-491 and miR-654) were predicted by bioinformatics to regulate the replication of H1N1 influenza virus genome through targeting PB1 gene and such inhibitions were then verified by biological assays (Song et al., 2010).

The biological function of human miR-21 has been relatively well studied in biomedical research. For example, in cardiac fibroblasts of heart failure, miR-21 enhanced ERK-MAP kinase activity through inhibiting expression of Spry1, and lowering miR-21 expression could inhibit interstitial fibrosis and attenuate myocardial dysfunction (Thum et al., 2008). Expression of miR-21 in tumor was found to be significantly higher than in normal tissues and miR-21 was found to be targeting several multiple tumor suppressor genes such as tropomyosin1 and lowering its expression could inhibit tumor growth, or tumor invasion and metastasis (Zhu et al., 2007, 2008). In this study, we explored the effect of host-encoded gga-miR-21 in chicken cells on IBDV replication and found that it had significant antiviral effects (Fig. 2).

In elucidating the mechanism of gga-miR-21 anti-IBDV activity, we first searched for gga-miR-21 target sequence in IBDV genome by bioinformatics and the 1713–1734nt in VP1 gene of IBDV was found. Then we confirmed with dual luciferase reporter system in DF-1 cells and DF-1 cells overexpressing gga-miR-21 or a control miRNA cel-lin-4. Following IBDV infection of the cell lines, IBDV replication was markedly lower in cells overexpressing gga-miRNA-21 than parental DF-1 cells or DF-1 cells overexpressing cel-lin-4 (Fig. 2). IBDV replication (by $TCID_{50}$ titration) and VP1 protein level (using Western blotting) in DF-miR-21 cells was dramatically lower than that in DF-lin-4 or DF-1 cells but VP1 mRNA level (using qRT-PCR assay) was not different (Fig. 5). The findings indicated that gga-miR-21 could suppress IBDV replication through down regulating IBDV VP1 expression at translational levels.

For chicken, more than 859 miRNAs have been identified so far (http://www.mirbase.org/), and only a few have been studied functionally. We have demonstrated in this study that gga-miR-21 inhibits IBDV replication through inhibiting VP1 mRNA translation via targeting a 22nt region of VP1 mRNA and not through degradation of VP1 mRNA. As VP1 is a RNA-dependent RNA polymerase responsible for both IBDV genome replication and mRNA synthesis (Liu and Vakharia, 2004; Lombardo et al., 1999; von Einem et al., 2004), VP1 protein level is closely related to IBDV replication and virulence (Boot et al., 2005). Therefore, the data can be interpreted that gga-miR-21 inhibits of VP1 protein synthesis, thereby weakens IBDV replication and lowers virus titers. As this 22nt VP1 sequence (5′-T*GATATCAG*G*GGCAAGCTG*-3′) is 100% (22/22) conserved among at least 30 other IBDV strains and shorter identical sequence were found in at least 70 other IBDV strains (BLAST search performed 06-21-2013), it is possbile that this regions might be essential for the RNA-dependent NA polymerase function of VP1 for virus replication. To our knowledge, there has been report on functional domains of IBDV VP1. Therefore, gga-miR-21 might target a key conserved region of IBDV VP1 which merit further study for VP1 function and this region might also serve as target for antiviral manipulations. Although antiviral drug is not common in poultry, but the principle of using chemically modified oligonucleotides for antiviral purposes has already been established, and parallel findings could be made to explore antivi-

ral functions of human miRNA and their target viruses. At this point a funtional connection between miR-21 in human cells and in chicken cells has not been made, but at least miR-21 has been confirmed to have a clear biological function in chicken cells during IBDV infection. As we have shown previoulsy that gga-miR-21 is significantly up-regulated in chick embryo fibroblasts and Bursa of Fabricius of SPF chickens after infection by different IBDV strains (Ouyang et al., 2012), the current study provided evidence to suggest chicken host up-regulate gga-miR-21 as a defense mechanism to fight back against IBDV by inhibiting virus replication. On the other had, as there are IBDV strains with VP1 containing shorter matching sequences to gga-mIR-21 target, it is possible those IBDV strains are less responsive to gga-miR-21 suppresion.

The methodology adopted in the current study – using a lentiviral vector system and DF-1 cells to generated DF-1 derived cells with stable exogenic miRNA expression, has proven useful for examining interactions of host miRNAs and IBDV components and we consider it has multiple merits. First, compared to plasmid transient expression system, miRNA overexpressing stable cell lines overcome disadvantages of the former such as low transfection efficiency, liposome-related toxicity and transient nature of miRNA expression. Secondly, there are currently two choices with stable miRNA overexpressing cell line generation with a lentiviral expression system: to express pri-miRNA (~520nt, including ~250nt on each side) and to express pre-miRNA. The current study chose to express the pri-miRNA sequence because pri-miRNA is much longer and better recognized by Drosha and Dicer enzymes for processing into mature miRNAs of nearly natural structures. Thirdly, lentivirus could infect dividing cells as well as non-dividing cells, and also has advantages such as efficient integration and expression (Sena-Esteves et al., 2004). DF-1 is one of the chicken embryo fibroblast cell lines. The cell line is more easily and stably cultured than primary cell cultures. In addition, DF-1 cells are excellent host for production of IBDV vaccine strain B87. The IBDV B87 could grow to very high titers in DF-1 and vial genome remains stable during many passages in this cell line. Both the virus (IBDV B87) and the cell line (DF-1) have been wide used by IBDV researches. In our experiments, the established DF-miR-21and DF-lin-4 cell lines were very stable in culture, and the cell lines expressed the desired miRNAs with high reproducibility and stability. The system used could be expanded for studying functions of other chicken miRNAs with IBDV.

In summary, the current study not only revealed the impact of gga-miR-21 overexpression on IBDV replication and its molecular mechanism, but also provided a sound technical approach for further understanding the molecular mechanisms of host miRNA against virus replication.

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