Antiviral Research 98 (2013) 66–75

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Borna disease virus encoded phosphoprotein inhibits host innate immunity by regulating miR-155

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ARTICLE INFO

Article history:
Received 2 November 2012
Revised 22 January 2013
Accepted 7 February 2013
Available online 18 February 2013

Keywords:
miR-155
SOCS
IFN
BDV
Persistent infection

ABSTRACT

It has been reported that the Borna disease virus (BDV) encoded phosphoprotein (P protein) can inhibit the activity of Traf family member-associated NF-kappaB activator (TANK)-binding kinase 1 (TBK-1), thus preventing the induction of type I interferon (IFN). However, the effects of microRNA on the regulation of BDV infection and the host's immune response have not been characterized. miR-155 was predicted to be complementary to the BDV P mRNA by RNAhybrid software. Here, we showed that miR-155 was down-regulated in BDV persistently infected human oligodendroglial (OL/BDV) cells and that the BDV P protein, but not the X protein, directly inhibited miR-155 expression in cells. When miR-155 was over-expressed, the inhibition of type I IFNs by BDV in cells was reversed, and the expression of type I IFNs was increased. When miR-155 expression was specifically blocked, cellular IFN expression and the induction of IFN by poly I:C treatment were suppressed. Furthermore, miR-155 promoted type I IFN production by targeting suppressor of cytokine signaling 1 (SOCS1) and SOCS3. Mutations in the nt1138–nt1158 region of SOCS3 abandoned the impact of miR-155 on the expression of SOCS3-enhanced green fluorescent protein (EGFP). The levels of BDV P mRNA and protein were significantly decreased in OL/BDV cells when miR-155 was over-expressed; however, miR-155-mutation did not affect the expression of BDV P-EGFP. Thus, BDV persistent infection inhibited the expression of type I IFNs through the suppression of miR-155, and miR-155 played an important immune regulatory role in BDV persistent infection.

1. Introduction

Innate immunity is the first line of defense against pathogenic microorganisms in the body (MacMicking, 2012). The type I interferons (IFNs) are important mediators of the innate immune response against viral infection (Haller et al., 2006). However, many viruses escape the innate immune response by different mechanisms to maintain a persistent infection (Weber et al., 2004).

Borna disease virus (BDV) is a non-segmented, negative-strand RNA virus that mainly establishes a persistent infection, which is closely associated with the inhibition of IFN expression by BDV. Unterstab et al. (2005) demonstrated that the BDV encoded phosphoprotein (P protein) interacts with Traf family member-associated NF-kappaB activator (TANK)-binding kinase 1 (TBK-1), a kinase of the retinoic acid-inducible gene-I (RIG-I)-dependent signaling pathway, to inhibit the induction of IFN. Furthermore, P protein can be phosphorylated by TBK-1 and competes with inducible transcription factor IFN regulatory factor 3 (IRF3) as a kinase substrate, indicating that this viral protein can reduce the activation of IFN genes (Unterstab et al., 2005). Recent *in vitro* studies have confirmed that the P protein inhibits the transcription factors involved in IFN induction. In addition, the study also found that the BDV P protein can attenuate the production and induction of type I IFNs (Peng et al., 2007).

Recent studies indicate that in addition to regulating viral replication through direct binding to the viral genome or to mRNAs, microRNAs (miRNAs) also regulate viral infection and the innate immune response. In latently Epstein–Barr virus (EBV)-infected cell lines, the viral latent membrane protein 1 (LMP1) protein negatively regulates the expression of IFN through the induction of miR-146a (Cameron et al., 2008). We previously showed that miR-122 induces the innate immune response in BDV persistently infected human oligodendroglial (OL/BDV) cells to regulate the interaction between the virus and the host's innate immune (Qian et al., 2010). Other studies have shown that the inducible miR-155

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http://dx.doi.org/10.1016/j.antiviral.2013.02.009

promotes type I IFN signaling in macrophages acutely infected with vesicular stomatitis virus (VSV), which then regulates the innate antiviral response through a positive feedback mechanism (Wang et al., 2010). miR-155 stabilizes EBV latent persistence through attenuation of NF-kappaB signaling by targeting IkappaB kinase epsilon (Lu et al., 2008). Here, we further investigated that BDV-encoded proteins inhibits host innate immunity by regulating microRNA.

We demonstrate that BDV-encoded P protein inhibits miR-155 expression, and miR-155 positively regulates the production of type I IFNs by suppressing the expression of suppressor of cytokine signaling 1 (SOCS1) and SOCS3. Therefore, we propose a new mechanism that BDV-encoded P protein inhibits host innate immunity by regulating miR-155.

2. Materials and methods

2.1. Cells culture and treatment

The OL and OL/BDV cells were offered by Professor Kazuyoshi Ikuta, Department of Virology, Osaka University, Japan. OL and OL/BDV cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% and 2% fetal bovine serum (FBS), respectively, and 100 U/ml penicillin/streptomycin, at 37 °C and 5% CO_2. OL and OL/BDV cells were treated with poly I:C (PIC, 100 μg/ml) (SIGMA, USA) for 4 h and harvested for analysis mRNAs of type I IFNs or BDV encoded proteins.

2.2. Plasmids

Plasmids for expressing shRNA targeting P mRNA of BDV (shRNA-424 and shRNA-582) were constructed by GenePharma (Shanghai, China). Two eukaryotic expression vectors for the p10 (X) and p24 (P) of BDV fused with enhanced green fluorescent protein (EGFP) were also prepared in this study. Briefly, the genes of X and P of BDV were obtained by polymerase chain reaction (PCR) amplification and cloned into the multiple cloning sites in the pEGFP-N1 (TaKaRa, Japan). IRF7 open reading frame (ORF) was inserted into the multiple cloning site of pmCherry-N1 (TaKaRa, Japan), and IRF7 fused with red fluorescent protein (RFP) mCherry could be expressed by pmCherry-IRF7. The structure of all plasmids used in this study had been confirmed by sequencing.

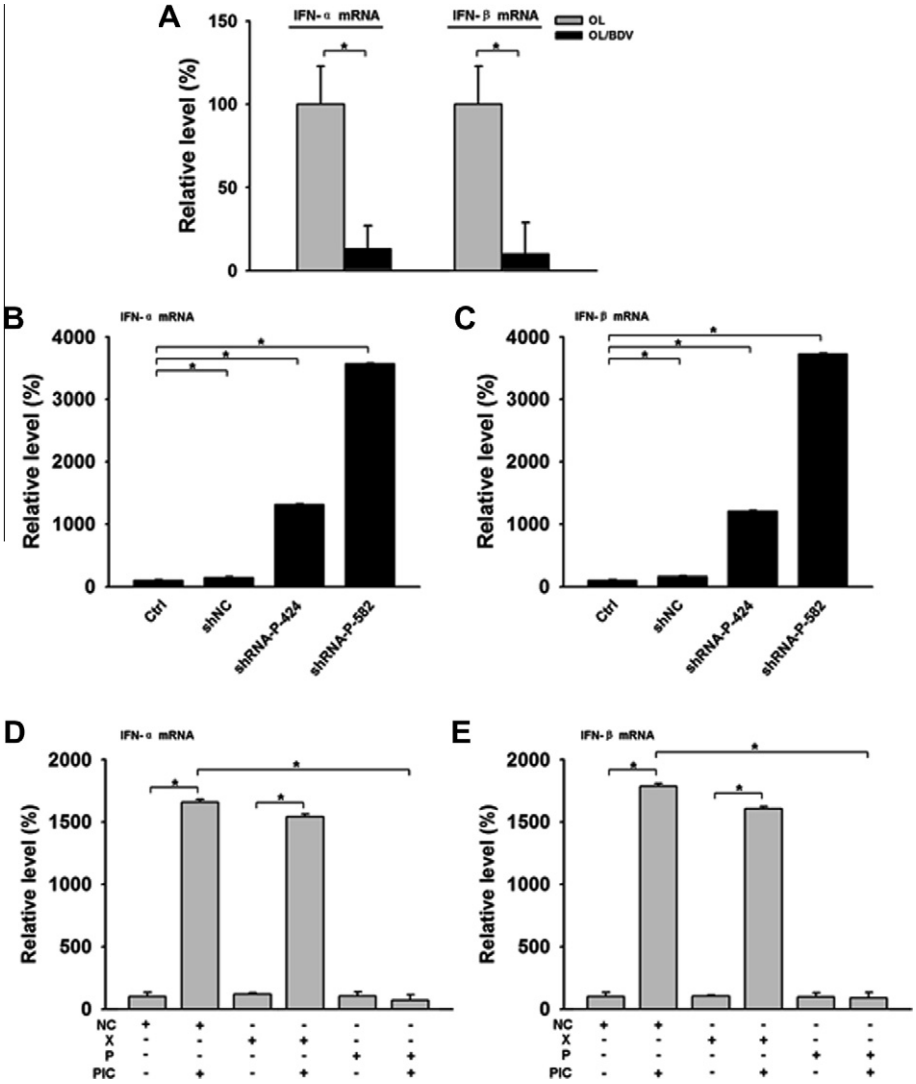

Fig. 1. The effect of BDV P protein on the expression of type I IFNs. (A) Detection of the expression of IFN-α and IFN-β mRNAs in OL and OL/BDV cells using real-time PCR. (B) and (C) Quantitation of IFN-α (B) and IFN-β (C) mRNAs in OL/BDV cells at 48 h post-transfection with shRNA-P-424 or shRNA-P-582 plasmid using real-time PCR. Untransfected cells (Ctrl) and the NC plasmid were used as controls. (D) and (E) At 24 h post-transfection of BDV X/P expression plasmids, OL cells were either treated with PIC for 4 h, and the induction of IFN-α (D) and IFN-β (E) mRNAs was detected by real-time PCR; the NC plasmid was used as a control. Data are presented as means ± SE and are representative of three independent experiments with similar results. $^{*}p < 0.05$.

The CDS and 3′UTR gene of SOCS3 (NM_003955.3) were obtained by PCR amplification and cloned into the multiple cloning sites in the pEGFP-C1 (wt-SOCS3). Overlapping PCR was used to mutate the putative miR-155 target in the SOCS3. The primers included primer pair A (A1: 5′-CCCAAGCTTGTCACCCACAGCAAGTTTCCCGCCGCCGGGATGAGCCG-3′, A2: 5′-GCTCTAGATTGACTTGGATTGGGATTTTGTTGAGTTCTTCAAGCATCTCC-3′; A1 underlined: Hind III restriction site; A2 underlined: Xba I restriction site), primer pair B (B1: 5′-CGCTCAGCTCCGTTGCTCATGGCACAAGCACAAGAAG-3′, B2: 5′-TGAGCAACGGAGCTGAGCGGCCTGCGTCCCCTCTCCCGACCCAT-3′). The pSOCS3-EGFP DNA, served as the template, was amplified with the primers A1 and B2, A2 and B1, respectively. The PCR products were purified and mixed to amplify with primers A1 and A2. The resultant plasmid contained the mutation in the nt1138–nt1158 of SOCS3 sequence (mu-1138-SOCS3). The mutation plasmid was verified by DNA sequencing.

2.3. Synthesis of miRNA and miRNA inhibitor

The recombinant plasmid pDC-316-EGFP-U6-miR-155 was synthesized by Genscript, USA, with miR-blank plasmid as control. The sense and antisense miR-155 mimics (5′-TTAATGCTAATCGTGATAGGGGT-3′ and 5′-ACCCCTATCACGATTAGCATTAA-3′), and the miR-mock were synthesized by GenePharma (Shanghai, China). The sequence of miR-155 inhibitor (anti-miR-155 oligonucleotide, AMO-155) used in this study (5′-ACCCCTATCACGATTAGCATTAA-3′) was the exact antisense copy of mature miRNA sequence for human miR-155. The sequence of the mismatched AMO-155 (AMO-155-mu served as negative control) carries ten mismatched nucleotides to miR-155 primarily (5′-CAAACTATCACGATTCTACGGCA-3′); the mismatched nucleotides are underlined. All the nucleotides in the AMO-155 and AMO-155-mu contained 2′-*O*-methyl modifications at every base were synthesized by Integrated DNA Technologies, USA. miR-155-mu mimics is the mutation of miR-155 targeting the nt11-nt31 of BDV P sequence (TTAATCGAATAGCACATAGGGGT).

2.4. Quantitative RT-PCR

Total RNA was isolated using TRIzol reagents (Invitrogen, USA) according to the manufacturer's instructions. 1 μg of RNA sample was reverse transcribed using reverse transcriptase (M-MLV, Promega, USA) in a standard protocol with oligo dT (TaKaRa, Japan), U6 RT primer (Qian et al., 2010) or miR-155 RT primer (5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACCCCT-3′). SYBR Green (TaKaRa, Japan) based real-time PCR was used. Fold variations between RNA samples were calculated by $2^{-\Delta\Delta ct}$ method after normalizing to the GAPDH mRNA or U6 RNA. The primers used in the experiments were shown as follows, miR-155 (F, 5′-GGGGTTAATGCTAATCGTGA-3′; R, 5′-CAGTGCGTGTCGTGGAGT-3′), SOCS3 (F, 5′-CAGCTCCAAGAGCGAGTACCA-3′; R, 5′-AGAAGCCGCTCTCCTGCAG-3′) (Tannahill et al., 2005); the primers of GAPDH, IFN-α, IFN-β, BDV P, and U6 were described previously (Qian et al., 2010).

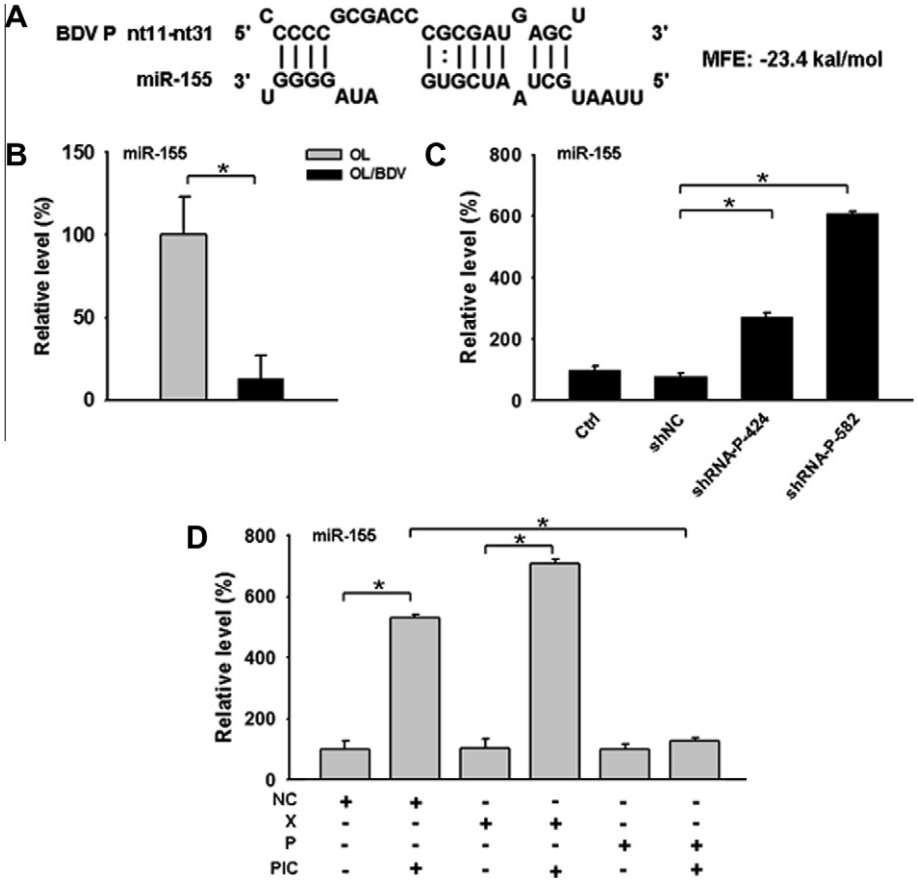

Fig. 2. Effect of BDV P and N proteins on the expression of miR-155. (A) Prediction of miR-155 binding site and MFE of the BDV P mRNA and miR-155. (B) Detection of the expression of miR-155 in OL and OL/BDV cells using real-time PCR. (C) Detection of miR-155 expression in OL/BDV cells using real-time PCR at 48 h post-transfection of shRNA-P-424 or shRNA-P-582 plasmid. Untransfected cells (Ctrl) and the NC plasmid served as controls. (D) At 24 h post-transfection of the BDV X/P expression plasmids, OL cells were treated with PIC for 4 h, and the induction of miR-155 was detected by real-time PCR; the NC plasmid was used as a control. Data are presented as means ± SE and are representative of three independent experiments with similar results. $^{*}p < 0.05$.

2.5. ELISA

The production of IFN-β in culture supernatant was measured using the VeriKine Human IFN-β ELISA kits (PBL interferon source, Piscataway, NJ) according to the manufacturer's instructions.

2.6. Quantitation of EGFP expression

SOCS3-EGFP expression in the pSOCS3-EGFP-transfected cells was analyzed at 32 h post-transfection using a fluorescence microscopy (Axiovert 200, Carl Zeiss, Gottingen, Germany) and a fluorescent spectrometer NanoDrop 3300 (Thermo, Rockford, IL). For fluorescence spectrometry, Hoechst 33342 (Invitrogen) was added to the culture medium at 37 °C with 5% CO_2 for 30 min to stain the nucleus 30 min before harvest. The proteins were extracted with the Pierce RIPA Buffer (Thermo, Rockford, IL) along with a protease inhibitor phenylmethylsulfonyl fluoride (PMSF) cocktail (Beyotime, Beijing, China). The SOCS3-EGFP fluorescence intensity was normalized to the Hoechst 33342 fluorescence intensity to eliminate variation in the cell populations of each sample.

2.7. Western blot analysis

Protein samples got from OL and OL/BDV cells were extracted by cell lysis buffer (Beyotime, China), separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The primary antibodies to BDV P, GAPDH (Santa Cruz, USA), β-actin (Santa Cruz, USA), SOCS1 and SOCS3 (Santa Cruz, USA) were used for detection. The intensities of reaction products were recorded with Western blot Chemiluminescence Reagent Plus and expressed as a ratio to GAPDH or β-actin.

2.8. Statistical analysis

Group data were expressed as mean ± SEM. Statistical analyses were carried out using SigmaStat (3.0) (Systat Software, Richmond, CA). The Student's *t*-test was used to evaluate the differences between two groups. A p value of <0.05 was taken as statistically significant.

3. Results

3.1. Inhibition of type I IFN expression by BDV and its encoded P protein

Unterstab showed that the BDV P protein inhibits the induction of IFN-β (Unterstab et al., 2005). We also demonstrated that the expression of IFN-α and IFN-β mRNAs in OL/BDV cells is significantly lower than in OL cells (Fig. 1A). Gene knockdown was performed using shRNA plasmids that specifically down-regulate the expression of BDV P mRNA, including shRNA-P-424 and shRNA-P-582 (Fig. S1A), and the non-targeting control shRNA-N1 (NC) which does not bind viral mRNAs. OL/BDV cells were transfected with the above plasmids, the expression of type I IFNs in each shRNA-treated group significantly increased compared to the NC group ($p < 0.05$) (Fig. 1B and C), indicating that the BDV P protein suppresses the expression of type I IFNs.

Protein expressions have the same levels when OL cells were transfected with BDV P and X plasmids (Fig. S1B). Treatment of cells with the PIC increased the expression of type I IFNs in OL cells. However, the BDV P protein, but not the X protein, significantly suppressed type I IFN induction by PIC treatment (Fig. 1D and E), indicating that the BDV P protein also inhibits the induction of type I IFNs in cells.

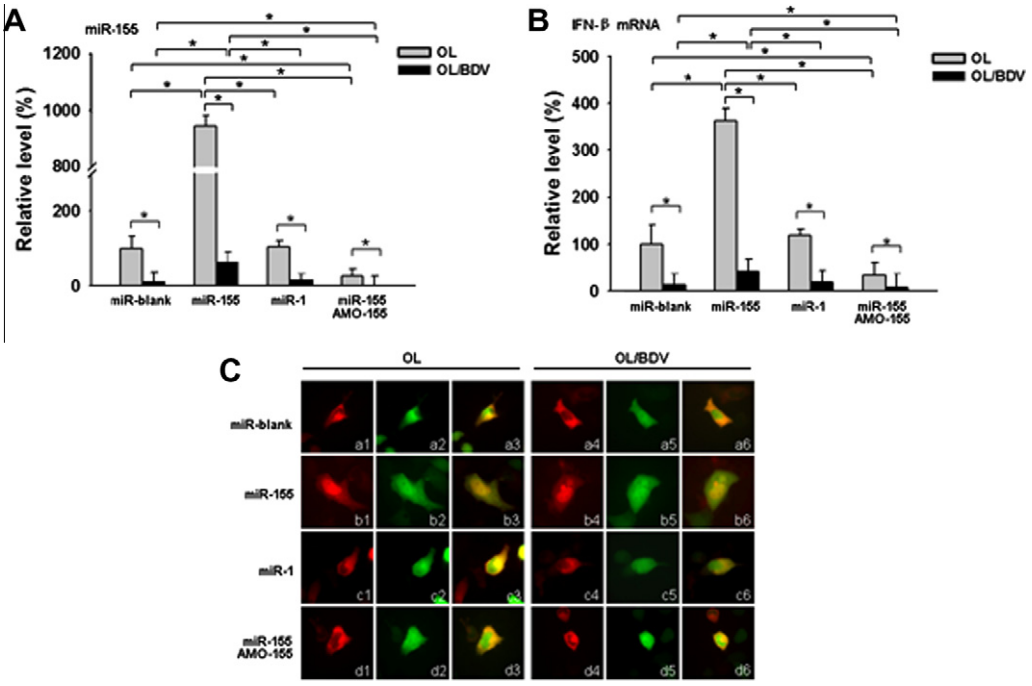

Fig. 3. Effects of over-expression of miR-155 on the production of type I IFNs. (A) and (B) OL and OL/BDV cells were transfected with miR-155 plasmid alone or co-transfected with miR-155 plasmid and AMO-155. The expression levels of miR-155 (A) and IFN-β (B) mRNAs were quantitated by real-time PCR at 48 h post-transfection; miR-blank plasmid was the negative control, and miR-1 plasmid was the non-targeting control. (C) The subcellular localization of mCherry-IRF7 in OL and OL/BDV cells treated as in (A) and (B) was observed by fluorescence microscopy. (a) miR-blank plasmid, (b) miR-155 plasmid, (c) miR-1 plasmid, and (d) miR-155 plasmid and AMO-155; (1) and (4) pmCherry-IRF7 plasmid, (2) and (5) EGFP-miR-blank/1/155 plasmid, (3) and (6) merged; (1–3) OL cells; (4–6) OL/BDV cells (×400). Data are presented as means ± SE and are representative of three independent experiments with similar results. $^{*}p < 0.05$.

3.2. Inhibition of miR-155 expression by BDV and the viral P protein

Using RNAhybrid 2.2 software, we predicted that miR-155 specifically binds to the 5′ ends of the BDV P (X60701.1) mRNA (Fig. 2A), suggesting a possible interaction between miR-155 and BDV P mRNA. Real-time PCR confirmed that the expression of miR-155 in OL/BDV cells was lower than in OL cells ($p < 0.05$) (Fig. 2B). Compared with the NC plasmid, the expression of miR-155 significantly increased after OL/BDV cells were transfected with shRNA constructs targeting BDV P mRNA (Fig. 2C). Furthermore, the BDV P protein significantly inhibited the induction of miR-155 by PIC treatment, whereas the X protein had no effect on the induction of miR-155 (Fig. 2D). Therefore, miR-155 expression is inhibited by BDV and its encoded P protein.

3.3. miR-155 directly induces type I IFNs in persistently virus-infected cells

miR-155 is a positive regulator of type I IFNs during an acute viral infection (Wang et al., 2010). Compared with cells transfected with miR-blank and miR-1 (non-targeting) expression vectors (Qian et al., 2010), the levels of miR-155 and IFN-β mRNA significantly increased in OL and OL/BDV cells transfected with miR-155 plasmid. However, when miR-155 plasmid was co-transfected with the miR-155-specific inhibitor of AMO-155, the expression of miR-155 and IFN-β mRNA in these two cell lines did not change significantly ($p < 0.05$) (Fig. 3A and B). The same result was obtained by ELISA (Fig. S2A). The expression of miR-155 did not affect the production of IFN-α mRNA (Fig. S2B). Pearson's correlation analysis showed a direct correlation between the IFN-β level and

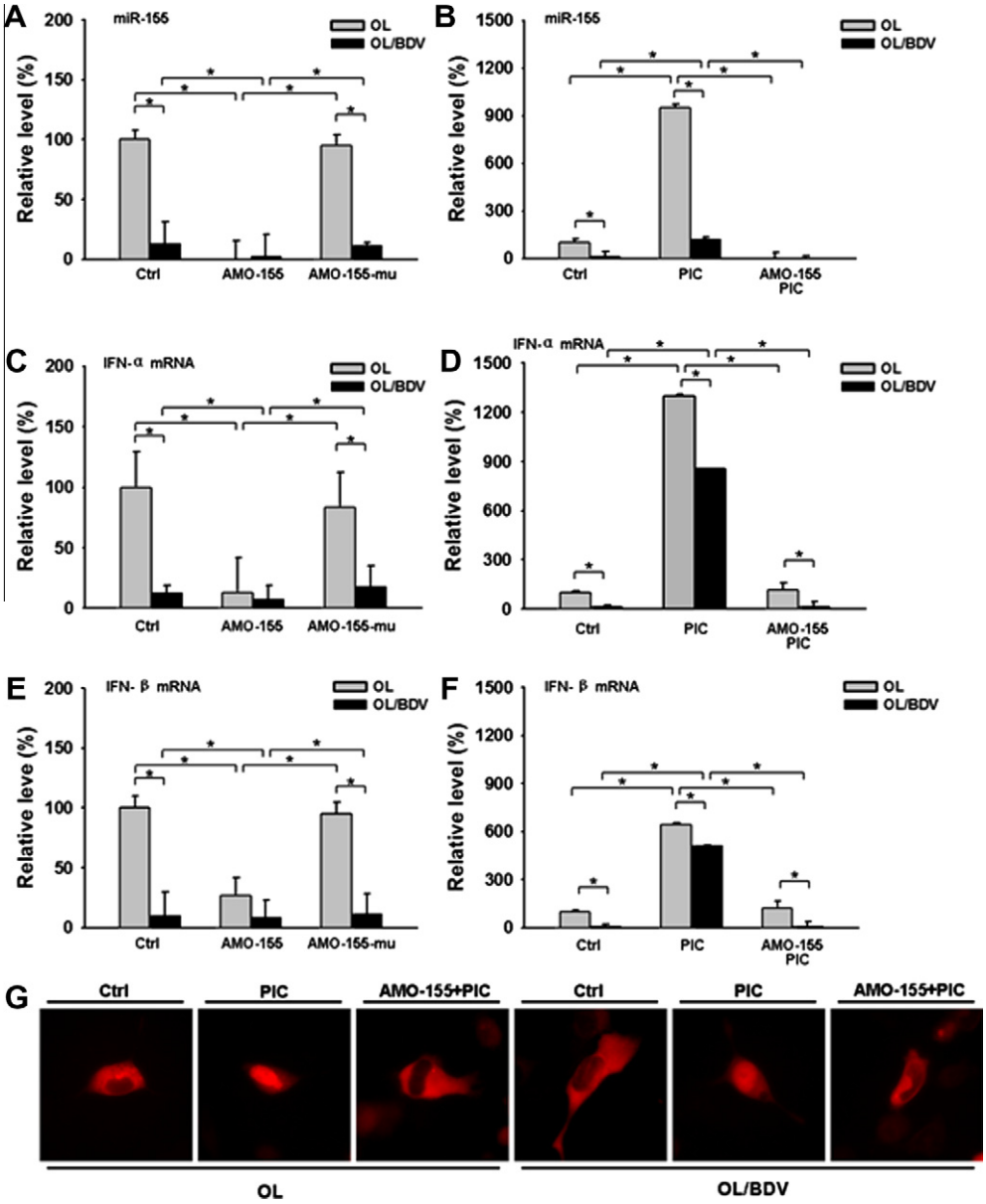

Fig. 4. Effects of low-expression of miR-155 on the production and induction of type I IFNs. (A), (C), and (E) The expressions of miR-155 (A), IFN-α (C), and IFN-β (E) mRNAs in OL and OL/BDV cells were quantitated by real-time PCR at 48 h post-transfection with AMO-155 or AMO-155-mu; normal cells (Ctrl) served as controls. (B), (D), and (F) At 24 h post-transfection with AMO-155, OL, and OL/BDV cells were treated with PIC or infected with CVB for 4 h. The expressions of miR-155 (B), IFN-α (D), and IFN-β (F) mRNAs were quantitated by real-time PCR. (G) The subcellular localization of mCherry-IRF7 in OL and OL/BDV cells treated as indicated was observed by fluorescence microscopy (×400). Data are presented as means ± SE and are representative of three independent experiments with similar results. *$p < 0.05$.

miR-155 over-expression in OL and OL/BDV cells (Fig. S2C and D). These results indicate that miR-155 promotes IFN-β production in OL and OL/BDV cells, and reverses the inhibition of IFN-β by BDV persistent infection.

When pmCherry-IRF7 was co-transfected with miR-blank or miR-1 plasmid for 48 h, the red fluorescence of IRF7 only localized in the cytoplasm of OL and OL/BDV cells. However, when co-transfected with miR-155 plasmid, IRF7 is translocated into the nucleus, whereas IRF7 remained in the cytoplasm when co-transfected with miR-155 plasmid and AMO-155 (Fig. 3C). These results further demonstrate that miR-155 promotes the production of type I IFNs.

OL and OL/BDV cells were transfected with AMO-155, the expression of miR-155 was significantly lower than AMO-155-mu-treated cells and untreated cells (Ctrl) (Fig. 4A). Furthermore, the expression of IFN-α and IFN-β mRNAs was significantly decreased with the down-regulation of miR-155 compared with control cells ($p < 0.05$) (Fig. 4C and E). These results indicate that the production of type I IFNs decreases when the expression of miR-155 is inhibited, which indirectly confirms that miR-155 can induce type I IFNs.

3.4. miR-155 regulates the induction of type I IFNs by IFN-inducing agents in persistently virus-infected cells

We next investigated the potential role for miR-155 in the induction of type I IFNs. OL and OL/BDV cells were pre-treated with AMO-155 for 24 h, followed by treatment with PIC for 4 h. In AMO-155-treated OL and OL/BDV cells, not only was the induction of miR-155 inhibited (Fig. 4B) but the induction of IFN-α and IFN-β by PIC was also significantly inhibited ($p < 0.05$) (Fig. 4D and F).

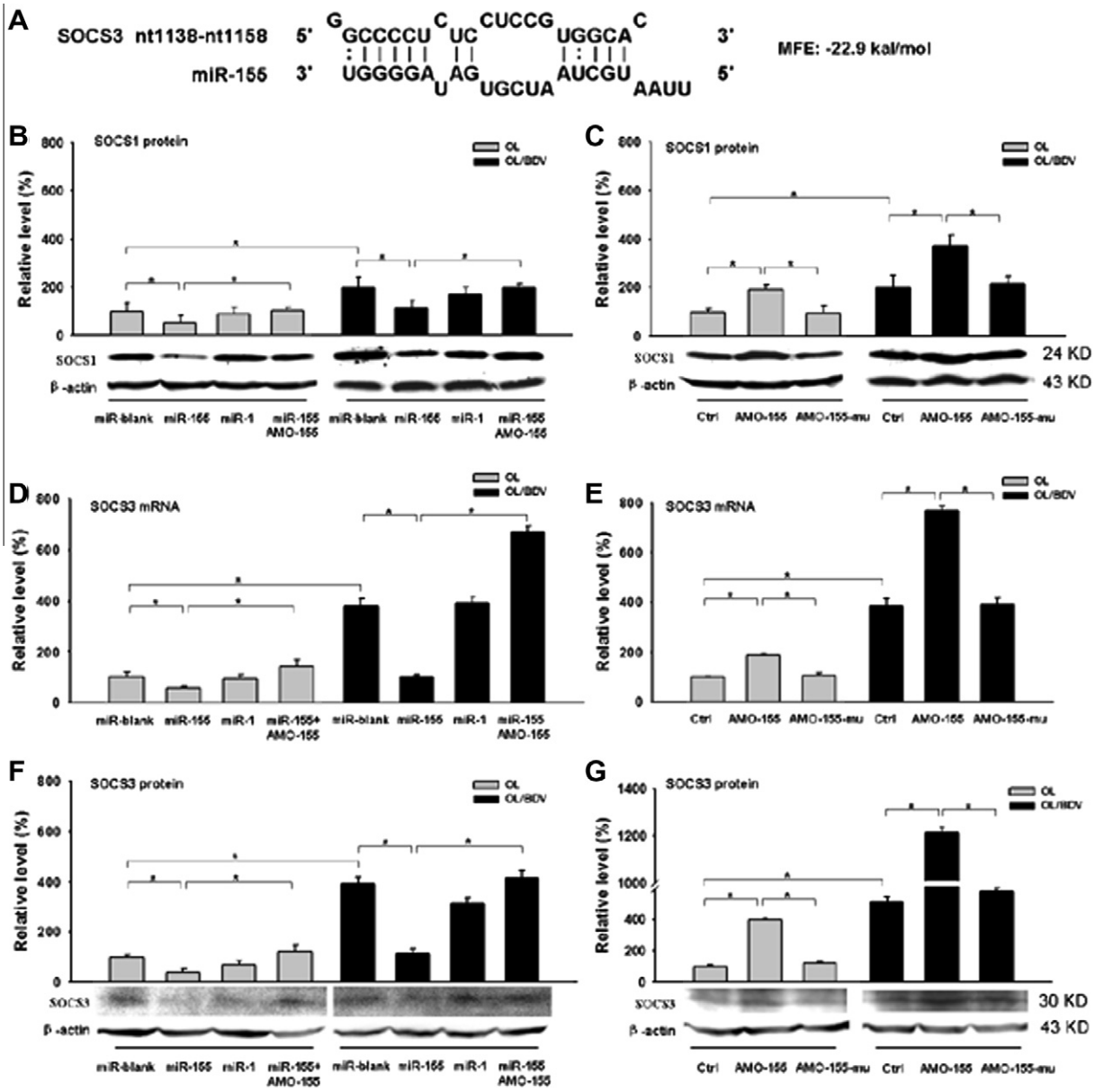

Fig. 5. Effects of miR-155 on the expressions of SOCS1 and SOCS3 mRNAs and proteins. (A) Prediction of miR-155 binding sites on SOCS3 mRNA using RNAhybrid software. (B) and (F) OL and OL/BDV cells were transfected with miR-155 plasmid alone or co-transfected with miR-155 plasmid and AMO-155. After 48 h, the expression of SOCS1 (B) and SOCS3 (F) proteins were visualized by Western blotting; miR-blank was used as a negative control, and miR-1 was used as a non-targeting control. (C) and (G) OL and OL/BDV cells were transfected with either AMO-155 or AMO-155-mu. The levels of SOCS1 (C) and SOCS3 (G) proteins were visualized at 48 h post-transfection by Western blotting; untransfected cells (Ctrl) were used as controls. (D) and (E) The expression of SOCS3 mRNA in cells treated as indicated was determined by real-time PCR. Data are presented as means ± SE and are representative of three independent experiments with similar results. $^{*}p < 0.05$.

In addition, after treatment with PIC, IRF7 is translocated from the cytoplasm into the nucleus of OL and OL/BDV cells. However, when miR-155 was specifically inhibited by AMO-155, treatment with PIC did not induce the nuclear translocation of IRF7 (Fig. 4G). These results demonstrate that the suppression of miR-155 expression blocks the induction of type I IFNs.

3.5. miR-155 positively regulates the production of type I IFNs through the inhibition of SOCS1 and SOCS3

Studies have confirmed that miR-155 inhibits the expression of SOCS1 (Jiang et al., 2010; Lu et al., 2009; Androulidaki et al., 2009); we also showed that miR-155 down-regulated the expression of SOCS1 protein (Fig. 5B and C). Furthermore, we found that SOCS3 (NM_003955.3) mRNA is predicted to contain a binding site for miR-155 (Fig. 5A). Compared with cells transfected with miR-blank or miR-1 plasmid, the expression of SOCS3 mRNA and protein significantly decreased in OL and OL/BDV cells transfected with miR-155 plasmid, whereas the expression of SOCS3 in cells co-transfected with miR-155 plasmid and AMO-155 was significantly higher than in cells transfected with miR-155 plasmid alone ($p < 0.05$) (Fig. 5D and F). The expression of SOCS3 mRNA and protein in OL and OL/BDV cells transfected with AMO-155 significantly increased compared with untransfected cells, whereas AMO-155-mu had no effect on the expression of SOCS3 (Fig. 5E and G).

To validate the inhibition of miR-155 on the SOCS3 expression, we introduced miR-mock and miR-155 mimics separately into OL cells. The wt-SOCS3 and mu-1138-SOCS3 was also co-transfected with the above miRNAs (Fig. 6A). The SOCS3-EGFP fusion protein expression was detected by counting SOCS3-EGFP-positive cells using a fluorescence microscope. miR-155 mimics reduced the EGFP expression of wt-SOCS3 comparing with miR-mock group; however, the SOCS3 protein expression in the cells co-transfected with miR-155 and mu-1138 were identical to each other ($p > 0.05$) (Fig. 6B and C). Moreover, the SOCS3-EGFP fluorescence intensities were consistent with that of SOCS3-EGFP-positive cell counts (Fig. 6D). Western blotting showed that the SOCS3-EGFP expression was also consistent with the SOCS3-EGFP fluorescence quantitation (Fig. 6E). These results indicate that miR-155 may positively regulate the production of type I IFNs not only through the suppression of SOCS1 but also by SOCS3.

3.6. Inhibition the expression of BDV P protein by miR-155

We next examined the effect of miR-155 on the expression of BDV proteins. The expressions of BDV P mRNA and protein were significantly decreased in OL/BDV cells transfected with the miR-155 plasmid, whereas the expression of BDV P mRNA and protein was not affected when AMO-155 was co-transfected with miR-155 plasmid. Moreover, the expression of BDV P mRNA was not altered after transfection with miR-1 plasmid or AMO-ctrl ($p < 0.05$)

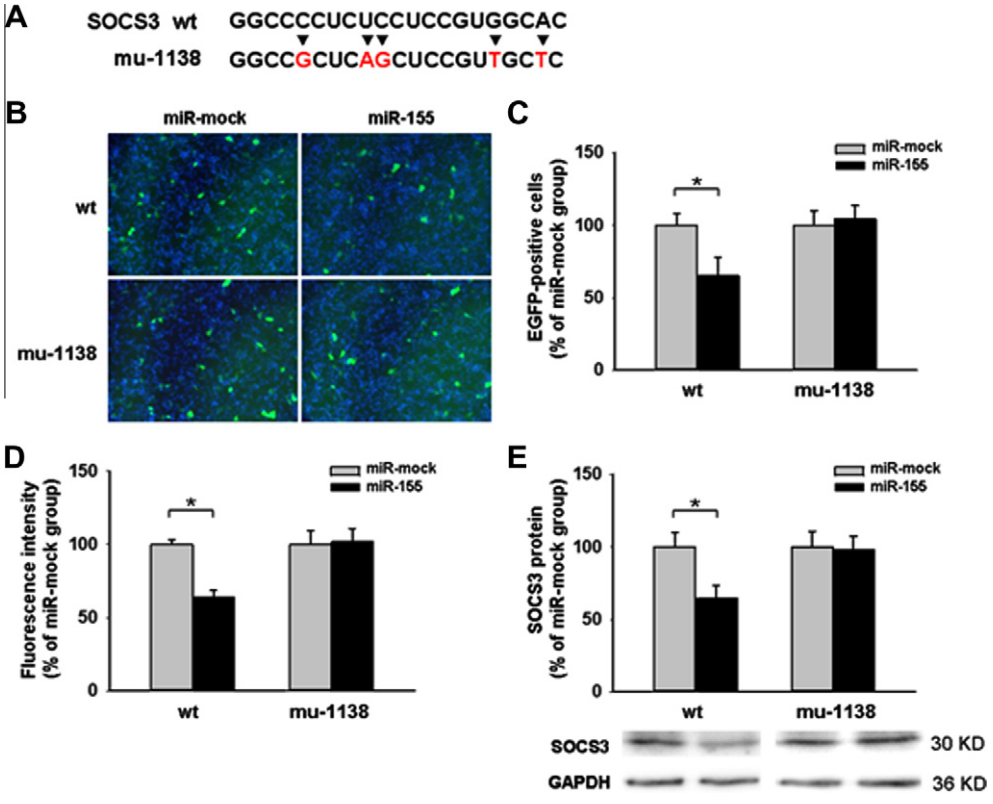

Fig. 6. Verification of miR-155 targets in the SOCS3 mRNA. (A) The mutated nucleotides in the putative target of SOCS3 (mu-1138). The red boxes are the mutated regions. For (B–E) miR-mock and miR-155 mimics were co-transfected into OL cells with wt-SOCS3-EGFP and mu-1138-SOCS3-EGFP plasmids, respectively. Hoechst 33342 was added into the culture medium to stain the nuclei half an hour before the detection. (B) SOCS3-EGFP expression in the treated cells was observed by fluorescence microscope at 32 h post-transfection. (C) The SOCS3-EGFP-positive cells counted and normalized to the number of nuclei in each view. The relative counts of the SOCS3-EGFP-positive cells of miR-155 mimics-treated group were calculated by normalizing to the miR-mock-treated group. (D) The SOCS3-EGFP fluorescence intensity measured by NanoDrop 3300. The SOCS3-EGFP intensity was normalized to the Hoechst 33342 intensity in each sample. The relative EGFP intensities of the miR-155-treated group were calculated by normalizing to the miR-mock-treated group. (E) The SOCS3-EGFP protein expression detected by Western blotting. The miR-mock was used as a normal control for the miR-155 mimics. Data are presented as means ± SE and are representative of three independent experiments with similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) $^{*}p < 0.05$.

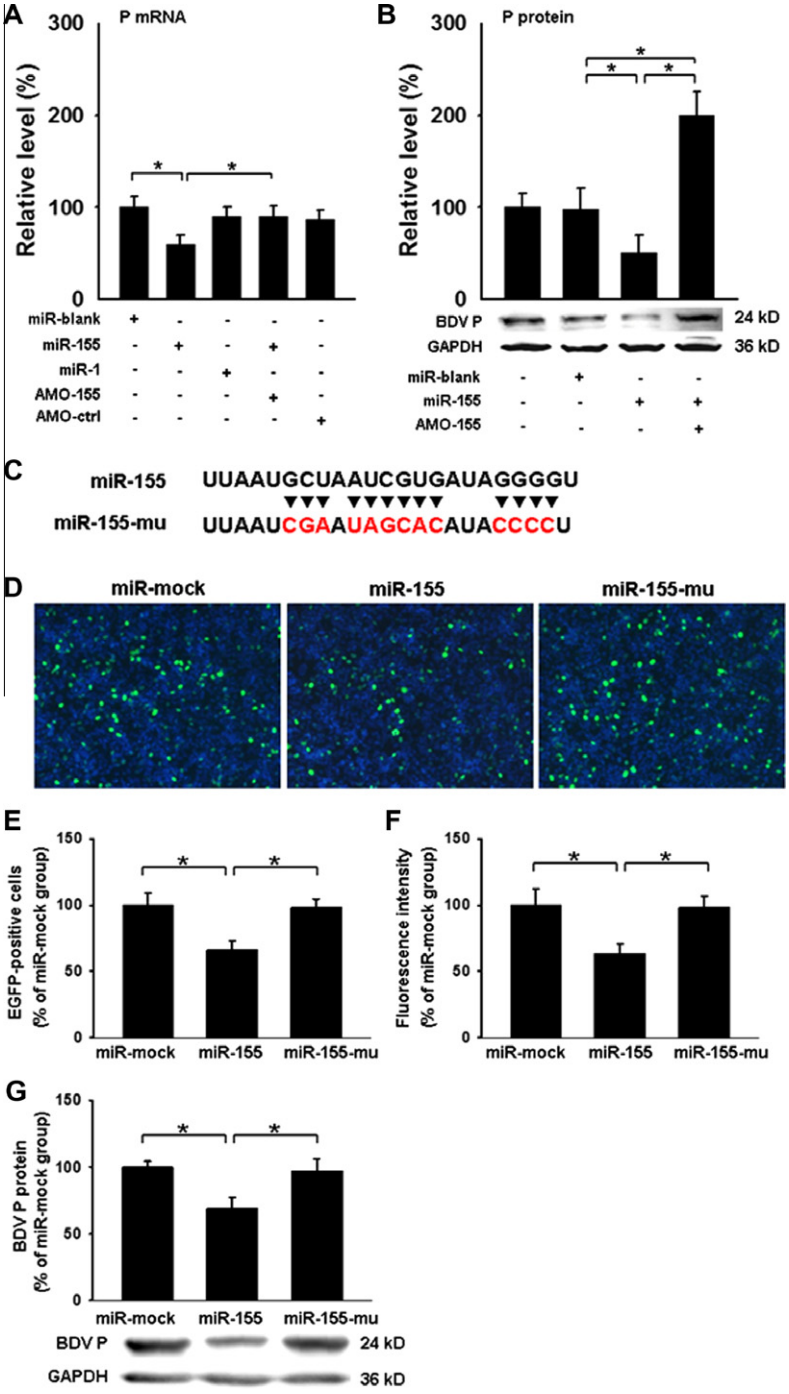

Fig. 7. Effect of miR-155 on the expression of BDV P mRNA and protein. (A) OL and OL/BDV cells were transfected with miR-155 plasmid alone or with miR-155 plasmid and AMO-155, and the expression of BDV P mRNA was measured by real-time PCR; miR-blank was used as a negative control, and the miR-1 plasmid and AMO-ctrl (miR-let-7d*) were non-targeting controls. (B) OL and OL/BDV cells were transfected with miR-155 plasmid alone or with miR-155 plasmid and AMO-155. The expression of BDV P protein compared with miR-blank-transfected cells was visualized by Western blotting; GAPDH served as an internal control. (C) The mutated nucleotides in the putative target of miR-155 (miR-155-mu). The red boxes are the mutated regions. For (D–G) miR-mock, miR-155 mimics and miR-155-mu mimics were co-transfected into OL cells with BDV P-EGFP plasmid. Hoechst 33342 was added into the culture medium to stain the nuclei half an hour before the detection. (D) BDV P-EGFP expression in the treated cells was observed by fluorescence microscope at 32 h post-transfection. (E) The BDV P-EGFP-positive cells counted and normalized to the number of nuclei in each view. (F) The BDV P-EGFP fluorescence intensity measured by NanoDrop 3300. (G) The BDV P-EGFP protein expression detected by Western blotting. Data are represented as means ± SE and are representative of three independent experiments with similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) $^{*}p < 0.05$.

(Fig. 7A and B). We co-transfected miR-mock, miR-155 mimics, and miR-155-mu mimics with BDV P-EGFP plasmid in OL cells (Fig. 7C). miR-155 could reduce the positive cell counts of BDV P-EGFP, miR-155-mu did not affect the positive cell counts (Fig. 7D and E). Moreover, the BDV P-EGFP fluorescence intensities were consistent with that of BDV P-EGFP-positive cell counts (Fig. 7F). Western blotting showed that the BDV P-EGFP expression was also consistent with the BDV P-EGFP fluorescence quantitation (Fig. 7G).

4. Discussion

miRNAs are small, endogenous, noncoding RNAs that are approximately 22–25 nucleotides in length. Typically, miRNAs target one or more mRNAs to regulate gene expression through the inhibition of translation or the cleavage of target mRNAs (Bartel, 2004; Shah et al., 2010; Davis-Dusenbery and Hata, 2010). Viruses participate in the regulation of miRNAs. HIV can effectively inhibit cellular miRNA clusters, including miR-17-5p, miR-17-3p, miR-18, miR-19a, miR-20a, miR-19b-1, and miR-92-1, which also inhibit HIV replication (Triboulet et al., 2007). The highly pathogenic avian influenza virus can infect humans, and human miR-507 and miR-136 can regulate the gene expression of this virus (Scaria et al., 2006). We also showed that the BDV P protein influence the expression level of miR-155 (Fig. 8).miRNAs also participate in the regulation of type I IFN production. Most studies have focused on miR-146a and miR-155. miR-146a regulates type I IFN production in a negative feedback loop. Hou showed that in murine macrophages, VSV increased miR-146a expression through the RIG-I-NF-κB pathway, and miR-146a targeted TRAF6, IRAK1, and IRAK2 to negatively regulate type I IFN expression (Hou et al., 2009). Wang showed that the inducible miR-155 promoted type I IFN signaling, STAT1 phosphorylation, and ISG15 and IP-10 expression by a positive feedback loop, which resulted in the inhibition of VSV replication (Wang et al., 2010). Our results show that the positive regulation of type I IFNs by miR-155 may be manifested in two ways: the direct promotion of type I IFN expression and participation in the induction of type I IFN production. miR-155 also has the function of reversing the inhibition of type I IFNs by BDV infection. Therefore, we propose a new mechanism for the virus–host interaction: in addition to the inhibition of IFN expression by BDV protein, BDV can also inhibit miR-155 expression, which results in the further attenuation of type I IFN production, escape from the innate immune response. This indicates a new target of the virus–host interaction as well as a new pathway of the inhibition of IFN by BDV.

Currently, at least three protein families are known to participate in the negative regulation of the type I IFN signaling pathway, including the SOCS, protein inhibitor of activated STAT (PIAS), and protein phosphatase (PTP) families. Previous studies have shown that miR-155 targets SOCS1 to promote tumorigenesis (Jiang et al., 2010; Lu et al., 2009; Androulidaki et al., 2009). Wang showed that the inducible miR-155 targets and inhibits SOCS1 expression in acutely VSV infected cells, thus promoting the downstream signaling of type I IFNs; SOCS1 activated STAT1/2 phosphorylation to exert its activity against acute viral infection through the RIG-I/JNK/NF-κB-dependent pathway, but not the TLR/MyD88-dependent pathway (Wang et al., 2010). In this study, we showed for the first time that in addition to inhibiting the SOCS1 pathway, miR-155 also inhibited the SOCS3 pathway to positively regulate type I IFNs in persistently virus infected cells (Fig. 8). We first found the target site of miR-155 on SOCS3 is nt1138–nt1158 region. Moreover, the promotion of miR-155 expression in persistently virus infected cells by exogenous IFN-α and IFN-β indicates that miR-155 and type I IFNs cross-activate one another and form a positive feedback loop (data not shown). Therefore, miR-155 can amplify the antiviral activity of the innate immune response, which provides a novel avenue for the antiviral activity of IFN. In addition to directly exerting its antiviral effect though protein induction, IFN may also do so indirectly via the induction of miR-155.

Studies have shown that upon viral infection, host miRNAs play a potential antiviral role; they can bind directly to the viral genome or target mRNAs to regulate viral replication. miR-28, miR-150, and miR-223 bind to the 3′ end of the human immunodeficiency virus (HIV) genome to inhibit viral replication (Huang et al., 2007). Our previous studies demonstrated that miR-122 targets BDV P and N mRNAs to inhibit viral transcription and translation (Qian et al., 2010). Here, we show that miR-155 contains binding sites for BDV P mRNA and inhibits BDV multiplication. Therefore, there is a mutual inhibition between miR-155 and BDV that maintains a persistent viral infection (Fig. 8).

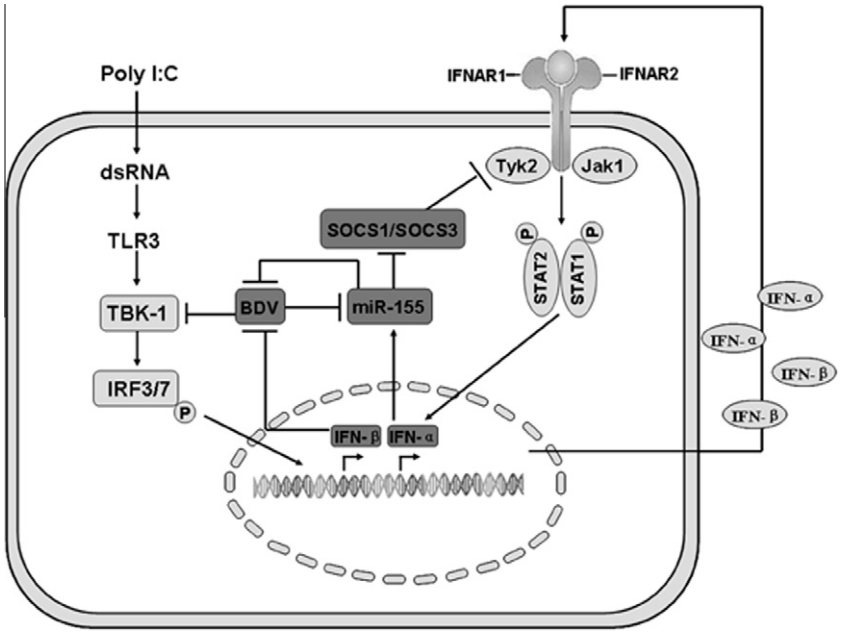

Fig. 8. BDV-encoded P protein inhibits host innate immunity by regulating miR-155. Upon viral infection, cells produce and secrete type I IFNs, and they activate IFN receptors (IFNAR) to produce large quantities of type I IFNs and IRF3/7. IRF3/7 plays an important role in the induction of type I IFNs. In this model, miR-155 up-regulates the production of type I IFNs through the inhibition of SOCS1 and SOCS3; type I IFNs also induces the expression of miR-155, which establishes a positive feedback loop. miR-155 inhibits BDV proliferation, and at the same time, BDV encoded P protein inhibits miR-155 expression. Therefore, BDV escapes the innate immune response by inhibiting miR-155, thus establishing a persistent infection.

In this study, we propose a new mechanism for the persistent infection of BDV. On the basis of mutual inhibition by BDV infection and type I IFNs, miR-155 participates in the establishment and stability of a persistent BDV infection. These observations suggest that host miRNA may target and participate in the regulatory network between persistent viral infection and the host immune response. These results provide a basis for the study of the regulatory roles of miRNAs in the persistent infections of other viruses.

Acknowledgments

This work was funded by grants from the National Natural Science Foundation of China (NSFC) (J1103609, J1210062, and 81202296), Foundation of Heilongjiang Provincial Education Department of China (11551238), Foundation of Heilongjiang Provincial Health Department of China (2009-207, 2009-216), Foundation of Heilongjiang Provincial Postdoctor of China (LBH-Z11086) and Natural Science Foundation of Heilongjiang Province of China (QC2012C058).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.02.009.

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