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Effective inhibition of infectious bursal disease virus replication in vitro by DNA vector-based RNA interference

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Abstract

Infectious bursal disease (IBD) leads to considerable economic losses for the poultry industry by inducing severe immunosuppression and high mortality in chickens. The objective of this study was to determine if RNA interference (RNAi) could be utilized to inhibit IBDV replication in vitro. We selected 3 short interfering RNA (siRNA) sequences (siVP1^{618}, siVP1^{1115}, and siVP1^{2571}) based on conserved regions in the *vp*1 gene of the infectious bursal disease virus (IBDV). When the Vero cells were transfected with siRNA, synthesized via in vitro transcription, and then infected with IBDV, siVP1^{2571} was discovered to be the most effective site for inhibiting IBDV replication. For long-term expression of siRNA and due to its suitability for large-scale preparation, the mouse U6 promoter was amplified using primers designed according to the siVP1^{2571} sequence. The resulting products were then subcloned into pEGFP-C1 to construct the shRNA expression vector pEC2571-shRNA. The shRNA-transfected Vero cells were then infected with IBDV. As compared to the control, the inhibitory rate in the pEC2571-shRNA-transfected group was 87.4%. Indirect immunofluorescence and real-time polymerase chain reaction (PCR) confirmed that VP1 expression decreased at both the protein and RNA levels as compared to that in the controls. The results presented here indicate that DNA vector-based RNAi could effectively inhibit IBDV replication in vitro.

Keywords: RNAi; siRNA; shRNA; Infectious bursal disease virus (IBDV); Virus replication

1. Introduction

Infectious bursal disease (IBD) is a highly contagious disease caused by the infectious bursal disease virus (IBDV). IBDV infection results in death and a severe decrease in the productivity of chickens and causes severe immunosuppressive disease via destruction of immature B-lymphocytes in the bursa of Fabricius in young chickens (Burkhardt and Muller, 1987; Müller, 1986; Sivanandan and Maheswaran, 1980). This damages the immune function and decreases the immune response to vaccines. IBD is mainly controlled via vaccine immunization. The extremely virulent IBDV strains reported in the 1980s usually lead to pathogenesis in vaccinated chickens (Chettle et al., 1989; Eterredossi et al., 2004). This challenged the prevention and treatment of IBD. IBDV is a double-stranded RNA (dsRNA) virus with a bisegmented genome, and it belongs to the genus *Avibirnavirus* of the family Birnaviridae (Dobos et al., 1979). The IBDV genome consists of two segments of double-stranded RNA, namely, A and B, and includes the 5′ non-encoding region, the encoding region, and the 3′ terminal non-encoding region (Hudson et al., 1986). Segment B encodes for a single open reading frame (ORF) that encodes for protein VP1 of molecular weight 90 kU which the virus RNA-dependent RNA polymerase (RdRp) associated with virus replication (Kibenge et al., 1997a).

The basic mechanism of RNA interference (RNAi) involves the closing or silencing of the gene expression of corresponding sequences by the dsRNA at the mRNA level; this is also known as gene silencing (Downward, 2004). Since this technique is a simple and effective tool, it has replaced the gene knockout approach and is widely used in the study of viral diseases, new gene screening, gene function assessment, and gene therapy (Brown et al., 1999; Cottrell and Doering, 2003; Lee et al., 2002). In mam-

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malian cells, RNAi is triggered by chemically or enzymatically synthesized RNA duplexes of 21–25 nt (Elbashir et al., 2001). Since the effect of short interfering RNAs (siRNAs) is generally transient in transfected animal cells, small RNA expression vectors have been developed to induce long-lasting RNA silencing in mammalian cells (Miyagishi et al., 2004; Pinkenburg et al., 2004; Rubinson et al., 2003; Yu et al., 2002; Zhao et al., 2003). These vectors use RNA polymerase III promoters to direct the synthesis of short hairpin RNA (shRNA) molecules, which are intracellularly processed into siRNA-like molecules. Vector-produced shRNA molecules are as effective for inhibiting gene expression as siRNAs generated in vitro. Hairpin expression vectors have been used to induce gene silencing in numerous studies on viruses (Brummelkamp et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2004; Paul et al., 2002; Yokota et al., 2003). For exploiting new methods to control IBD, we hypothesize that RNAi may provide effective protection against IBDV. In this study, we identified the target sites of the IBDV VP1 gene, screened the effective siRNA via in vitro transcription, constructed the shRNA expression vector, and investigated its inhibitory effect on IBDV replication in vitro.

2. Materials and methods

2.1. Selection of target sites and siRNA synthesis

Based on the *vp*1 gene sequence of the IBDV Gx strain (GenBank accession number: AY705393), 3 siRNA sequences (siVP1^{618}, siVP1^{1115}, and siVP1^{2571}) were selected using the siRNA Target Finder and Design Tool available at http://www.ambion.com/ (Fig. 1A). Their specificity was verified by conducting a BLAST search. Additionally, a non-specific siRNA sequence was set as the control (siRNA^{Con}). The siRNA ends were linked to T7 promoter sequences, according to the instruction manual of provided with the T7 RiboMAX^{TM} Express System (Promega, Madison, WI, USA) (Table 1). All the siRNAs were synthesized and stored at −70 °C.

2.2. Construction of shRNA-expressing vectors

Based on the siRNA screening results, the primers were designed according to the siRNA sequence (siVP1^{2571}) that demonstrated the best inhibitory effect. The universal forward

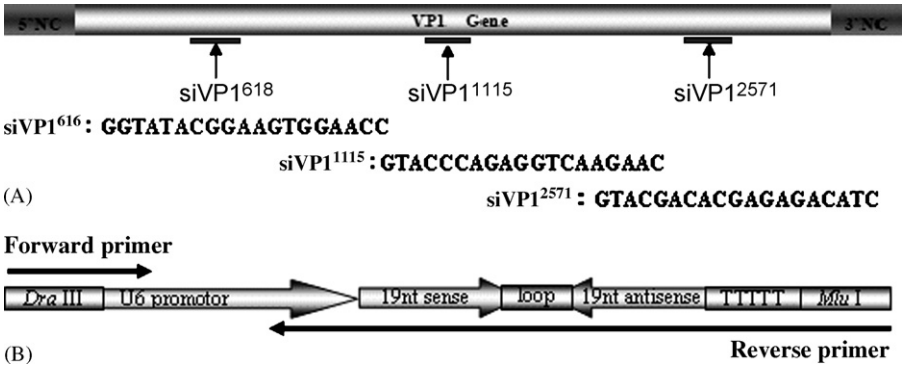

Fig. 1. Target small interference RNAs (siRNAs) and the polymerase chain reaction (PCR)-based strategy for construction of short hairpin RNA (shRNA) expressing vectors. (A) Genomic structure of the Infectious Bursal Disease Virus (IBDV) and the position of target siRNAs siVP1^{618}, siVP1^{1115}, and siVP1^{2571}. (B) PCR-based strategy for the construction of shRNA-expressing vectors. With regard to the forward primer, the arrow represents the sequences included based on the mouse U6 promoter and *Dra*III site. The reverse primer includes sequences complementary to the mouse U6 promoter, hairpin loop, 5 adenosines, and the *Mlu*I site.

Table 1
siRNA sequences

Name	Sequences
siVP1^{618}-1:	5′-GGATCCTAATACGACTCACTATA**GGTATACGGAAGTGGAACC**-3′
siVP1^{618}-2:	5′-AA**GGTTCCACTTCCGTATACC**TATAGTGAGTCGTATTAGGATCC-3′
siVP1^{618}-3:	5′-GGATCCTAATACGACTCACTATA**GGTTCCACTTCCGTATACC** -3′
siVP1^{618}-4:	5′-AA**GGTATACGGAAGTGGAACC**TATAGTGAGTCGTATTAGGATCC-3′
siVP1^{1115}-1:	5′-GGATCCTAATACGACTCACTATA**GCACATGGCTCACCAAGAC** -3′
siVP1^{1115}-2:	5′-AA **GTCTTGGTGAGCCATGTGC**TATAGTGAGTCGTATTAGGATCC-3′
siVP1^{1115}-3:	5′-GGATCCTAATACGACTCACTATA**GTCTTGGTGAGCCATGTGC** -3′
siVP1^{1115}-4:	5′-AA **GCACATGGCTCACCAAGAC**TATAGTGAGTCGTATTAGGATCC-3′
siVP1^{2571}-1:	5′-GGATCCTAATACGACTCACTATA**GTACCCAGAGGTCAAGAAC** -3′
siVP1^{2571}-2:	5′-AA **GTTCTTGACCTCTGGGTAC**TATAGTGAGTCGTATTAGGATCC-3′
siVP1^{2571}-3:	5′-GGATCCTAATACGACTCACTATA**GTTCTTGACCTCTGGGTAC** -3′
siVP1^{2571}-4:	5′-AA**GTACCCAGAGGTCAAGAAC**TATAGTGAGTCGTATTAGGATCC-3′
siRNA^{CON} -1:	5′-GGATCCTAATACGACTCACTATA**GTACGACACGAGAGACATC** -3′
siRNA^{CON} -2:	5′-AA **GATGTCTCTCGTGTCGTAC**TATAGTGAGTCGTATTAGGATCC-3′
siRNA^{CON} -3:	5′-GGATCCTAATACGACTCACTATA**GATGTCTCTCGTGTCGTAC** -3′
siRNA^{CON} -4:	5′-AA**GTACGACACGAGAGACATC**TATAGTGAGTCGTATTAGGATCC-3′

Notes: Sequences 1 and 2 are complementary sequences; sequences 3 and 4 are complementary sequences; the bold nucleotides comprise the siRNA sequence; the underlined nucleotides indicate the T7 RNA polymerase promoter sequence.

primer (pEGFPC1U6Dra) complementary to the 5′ end of the mouse U6 promoter was used. The sequence used was TAT*CACGTAGTG*GATC CGACGCCGCCATC. The *Dra*III restriction site was introduced in the 5′ end (underlined). The sequence of reverse primer (pEGFPC1U6Mlu_2571) was 5′GAC*ACGCGT*AAAAA**GTACCCAGAGGTCAAGAAC**tc-tcttgaa**GTTCTTGACCTCTGGGTAC**AAACAAGGCTTTT-CTCCAA 3′. The sequence included *Mlu*I site in the 5′ end (italics), siVP1^{2571} sequence, reverse complementary sequence (bold), and the loop sequence (lowercase). Simultaneously, an irrelevant shRNA expression vector was established. The following reverse primer was used (pEGFPC1U6Mlu_Con): 5′GAC*ACGCGT*AAAAA**GTACGACACGAGAGACATC**tc-tcttgaa**ATGTC TCTCGTGTCGTAC**AACAAGGCTTTTC-TCCAA 3′. All the primers were synthesized by Invitrogen Shanghai Co. The U6 promoter was amplified using pBS-U6 (a gift of Dr. Guangchao Sui, Harvard Medical School) as a template and the abovementioned primers (Sui et al., 2002). The PCR products were digested by *Mlu*I/*Dra*III and subcloned into the pEGFP-C1 vector, resulting in generation of the shRNA expression vector (Fig. 4). The recombinant vector was confirmed by restriction enzyme digestion, PCR, and sequence analysis. The positive plasmids were named pEC2571-shRNA and pECCon-shRNA. The plasmids used for transfection were purified using the QIAGEN plasmid Midi Kit (Qiagen, Chatsworth, CA).

2.3. Cell culture, transfection, and virus infection

The African green monkey kidney cell line Vero was grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Inc., San Diego, CA, USA) containing 10% fetal bovine serum (FBS), 170 mM penicillin, and 40 mM streptomycin at 37 °C in a humidified atmosphere containing 50 mL/L CO_2. One day before transfection, the Vero cells were seeded in 12-well plates (60–80% confluence) and transfected with siRNA (1.0 μg) or pEC2571-shRNA (1.6 μg) by using Lipofectamin^{TM}2000 (Invitrogen, Inc.) according to the manufacturer's instructions. This concentration was determined to be optimal in preliminary experimentation. Specificity of the inhibition was confirmed by transfecting the cultures with pEGFP C1 empty vector and the non-specific vector pECCon-shRNA. Four hours post-transfection, the cells were infected with IBDV Gx isolate (Wang et al., 2004) at a multiplicity of infection (MOI) of 0.01. The cultures were then incubated at 37 °C, 50 mL/L CO_2 in a humidified incubator for 1 h, at which point the culture medium was replaced with fresh DMEM containing 4% FBS. To determine transfection efficiency, we monitored the GFP fluorescence intensity of transfected cells on an inverted fluorescent microscope. After 60 h of incubation, the plates were freeze-thawed 3 times, and culture supernatants were collected for virus titration and real-time PCR analysis.

2.4. Virus plaque

A chicken embryo fibroblast (CEF) was prepared by standard procedures. An almost confluent monolayer of the CEF was infected with the virus at different concentrations (10^{-1} to 10^{-7}, dilutions used in duplicate). Each well was inoculated with 0.5 mL virus and incubated at 37 °C for 1 h. After the diluted virus solution was removed, the plate was covered with 4 mL 1% low-melting-point agar and then incubated in a 37 °C incubator; 48 h after incubation, the plate was covered with 4 mL 1% low-melting-point agar containing 1% neutral red. The plate was then incubated overnight at 37 °C for observing and counting the plaques.

2.5. Indirect immunofluorescence

The Vero cells were transfected with the shRNA expression vector. The cells were infected with IBDV 6 h after transfection. At 60 h after infection, the medium was discarded, and the cells were washed twice with pre-cold PBS (137 mM NaCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl; pH 7.4). Anhydrous ethanol (100 μL) was added to each well, and the cells were fixed at room temperature for 15 min. After washing with PBS, 150 μL anti-IBDV VP1 monoclonal antibody (4D4) ascites was added to each well, and the plates were incubated at 37 °C for 1 h. The cells were washed 5 times with PBS; 30 μL rhodamine (TRITC)-rabbit anti-mouse IgG enzyme conjugate (1:100) was then added, and incubation was performed at 37 °C for 45 min followed by washing (5 min for each wash) 5 times with PBS in the dark. The cells were observed under a fluorescence microscope (Leica, HC). Cells that were not transfected and infected were set as controls.

2.6. RNA extraction and reverse transcription (RT) and real-time PCR analysis

At 60 h after infection, total RNA was isolated from the cells by using the Trizol (Invitrogen, Inc.) reagent according

Table 2
The sequences of the primer used for real-time PCR

Primers	Sequences	Use	Size
B5	5′-AGGATACGATCGGTCTG -3′	VP1 standard preparation	1384 bp
B6	5′-ATTATGTCTTTGAAGG -3′		
VP1-RT1	5′-TATCTAGAGGGGCCCCCGCAGGCGAAG -3′	Real-time PCR	143 bp
VP1-RT2	5′- CCGGATTATGTCTTTGAAGCC -3′		
β- actin-U	5′ -GAGAAATTGTGCGTGACATCA -3′	Standard preparation and Real-time PCR	282 bp
β- actin-L	5′-CCTGAACCTCTCATTGCCA -3′		

to the manufacturer's instruction. cDNA was synthesized by reverse transcription. One microliter of reverse transcription reaction mixture was used for real-time PCR by using gene-specific primers (Table 2) and RealmasterMix (SYBR Green) (TIANGEN, Beijing, China). All reactions were done in a 25 μL reaction volume. The reaction was then performed at 95 °C for 10 min, followed by 40 cycles for 94 °C for 10 s, 60 °C for 15 s, 72 °C for 20 s, and 78 °C for 15 s. The target mRNAs in samples were quantified by comparison with a standard curve derived from known amounts plasmids of target gene. Quantitative expression was normalized using an internal control (β-actin). Amplification and detection of samples were performed with the Rotor-gene 3000 detection system (Gene Company Limited).

3. Results

3.1. Selection of effective siRNA

siRNA was transcribed in vitro using the RiboMAX™ Express RNAi System according to the manufacturer's protocol (Promega, Inc.). The siRNA concentration was determined by 1.5% agarose gel electrophoresis. The concentrations of siVP1^{618}, siVP1^{1115}, siVP1^{2571}, and siRNA^{con} were 416 ng/μL, 503 ng/μL, 369 ng/μL, and 559 ng/μL, respectively.

The Vero cells were transfected with siRNAs for 6 h and were then inoculated with IBDV to test the effect of siRNAs on IBDV replication. The cell suspension harvested from each experimental group was used for the virus plaque assay in order to determine the virus titer. As shown in Fig. 2, the virus titer of the siRNA^{Con} group was 3×10^4 pfu/mL, and those for the siVP1^{618}, siVP1^{1115} and siVP1^{2571} groups were 4.8×10^3, 4×10^3 and 2×10^3 pfu/mL, respectively. As compared to the siRNA^{Con} group, the inhibitory rates for IBDV replication of the siVP1^{618}, siVP1^{1115}, and siVP1^{2571} groups were 84%, 86.7%, and 93.3%, respectively.

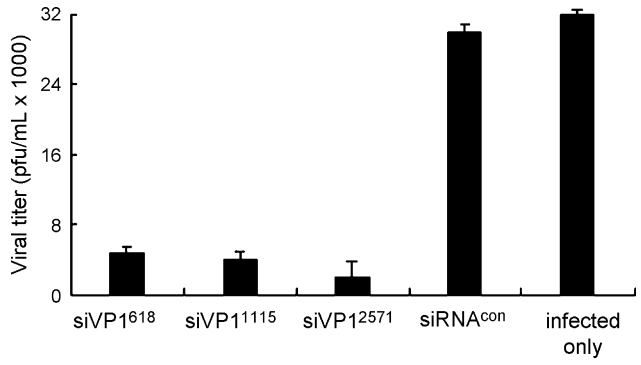

Fig. 2. siRNAs interfere with IBDV production in Vero cells. Supernatants collected from the 60 h IBDV-infected cells were serially diluted 10-fold and were used for the virus plaque assay to determine the virus titer. Virus titers are represented as PFU/mL × 1000. The data shown represents the mean value for three separate experiments; standard deviations indicated by error bars.

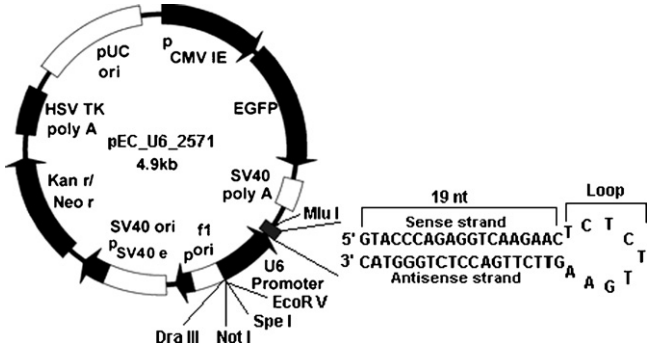

Fig. 3. Map of the pEC2571-shRNA expression vector. siVP1^{2571} sequence was expressed by shRNA expression plasmid. The hairpin structures of siRNA were formed after transcription, which would interfere with the targeted mRNA.

3.2. Effect of the shRNA expression vector on IBDV replication

3.2.1. shRNA expression vector construction

The shRNA expression cassette (U6 promoter and siVP1^{2571}) was subcloned into the pEGFP-C1 vector between the restriction sites 1642 (*Mlu*I)–1872 (*Dra*III). The shRNA expression cassette was inserted in the reverse direction (Fig. 3). Downstream primers contained the siVP1^{2571} sequence (Fig. 1B), and the irrelevant control was simultaneously established (upstream primer contained the siRNA^{con} sequence). PCR was performed using pBS-U6 (containing the mouse U6 promoter sequence) as the template. The PCR products were recovered from the agarose gel and digested with *Mlu*I/*Dra*III, followed by subcloning into the *Mlu*I/*Dra*III-digested pEGFP-C1 vector. The recombinant vector was confirmed as positive by *Mlu*I/*Dra*III digestion, *Eco*RI digestion, PCR, and sequencing.

3.2.2. shRNAs transcribed from DNA vectors inhibited IBDV replication

The ability of shRNA to inhibit IBDV replication was determined by the virus plaque assay for the infected cells that had been pre-transfected with the shRNA expression vectors. The results revealed that the virus titer for the siVP1^{2571} transcribed in vitro, pEC2571-shRNA, pECCon-shRNA, and the pEGFP-C1 empty vector pre-transfected Vero cells were 2.1×10^3, 3.3×10^3, 3.2×10^4, and 3.4×10^4 pfu/mL. Infection control was 3.5×10^4 pfu/mL, respectively. As compared to the empty vector controls, the inhibitory rate of the pEC2571-shRNA transfection group was 87.4% (Fig. 4).

3.2.3. Indirect immunofluorescence assay

To investigate the effect of the shRNA-expressing vectors for inhibiting VP1 protein expression, an indirect immunofluorescence assay was performed using the anti-VP1 protein MAb at 60 h post-infection. We monitored the GFP fluorescence intensity of transfected cells on an inverted fluorescent microscope (approximately 70%, data not shown) to determine transfection efficiency. The results revealed that the number of fluorescent positive cells was significantly decreased in the pEC2571-shRNA-tranfected group. In contrast, the infected cells in the wells pre-transfected with the pECCon-shRNA or pEGFP-C1

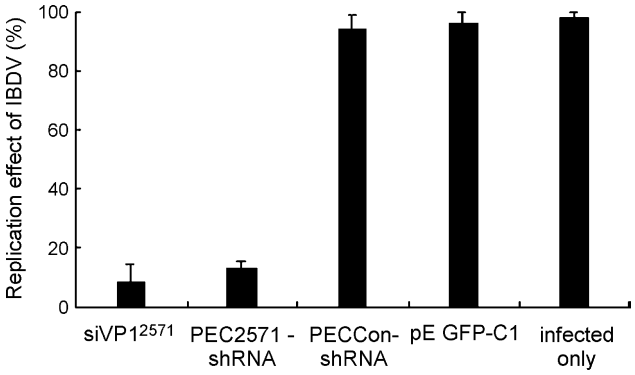

Fig. 4. siRNAs interfere with IBDV production in Vero cells. Supernatants collected from the 60 h IBDV-infected cells were serially diluted 10-fold and the virus titres were determined by the virus plaque assay. The data shown indicates the replication effect of IBDV after transfection with the shRNA-expressing vectors. The data shown represents the mean value for three separate experiments; standard deviations indicated by error bars.

vector had equivalent or a slightly lower number of VP1 protein-positive cells as compared to the controls (Fig. 5).

3.2.4. Real-time PCR results

In order to test the inhibitory effect of the shRNA expression vector on the VP1 protein at the mRNA level, VP1 RNA was quantitatively detected using SYBR Green I Real-time PCR, and β-actin was used as the internal control. VP1 mRNA levels in the siVP1^{2571} transcribed in vitro and pEC2571-shRNA-treated cells were 5.76E + 03 and 7.98E + 03. As compared to the cells without infection (8.58E + 04), the expression levels decreased to 93.3% and 90.7%. However, the VP1 mRNA levels in the pECCon-shRNA and pEGFP-C1 vector-treated cells were 6.83E + 04 and 8.18E + 04, indicating that there was no significant decrease. The mRNA levels of β-actin (the average β-actin mRNA levels in cells treated with siRNA^{2571}, pEC2571-shRNA, pEN-shRNA, the pEGFP-C1 vector, infection only and normal cells were 3.15E + 05, 2.95E + 05, 3.26E + 05, 3.01E + 05, 3.11 E + 05 and 3.21E + 05, respectively) suggested that the reduction in the VP1 mRNA was not a result of poor transfection, non-specific inhibition, or toxicity, because the average mRNA levels of β-actin of the experimental cells did not significantly decrease as compared to that in the control cells. In addition, the suppressive effect was found to be gene specific because the inhibitory effects of the empty vector and non-specific shRNA expression vector pECCon-shRNA were negligible (Fig. 6).

4. Discussion

RNAi can significantly suppress gene expression by inducing degradation of the targeted mRNA and by down-regulating the level of the corresponding proteins when exogenous dsR-

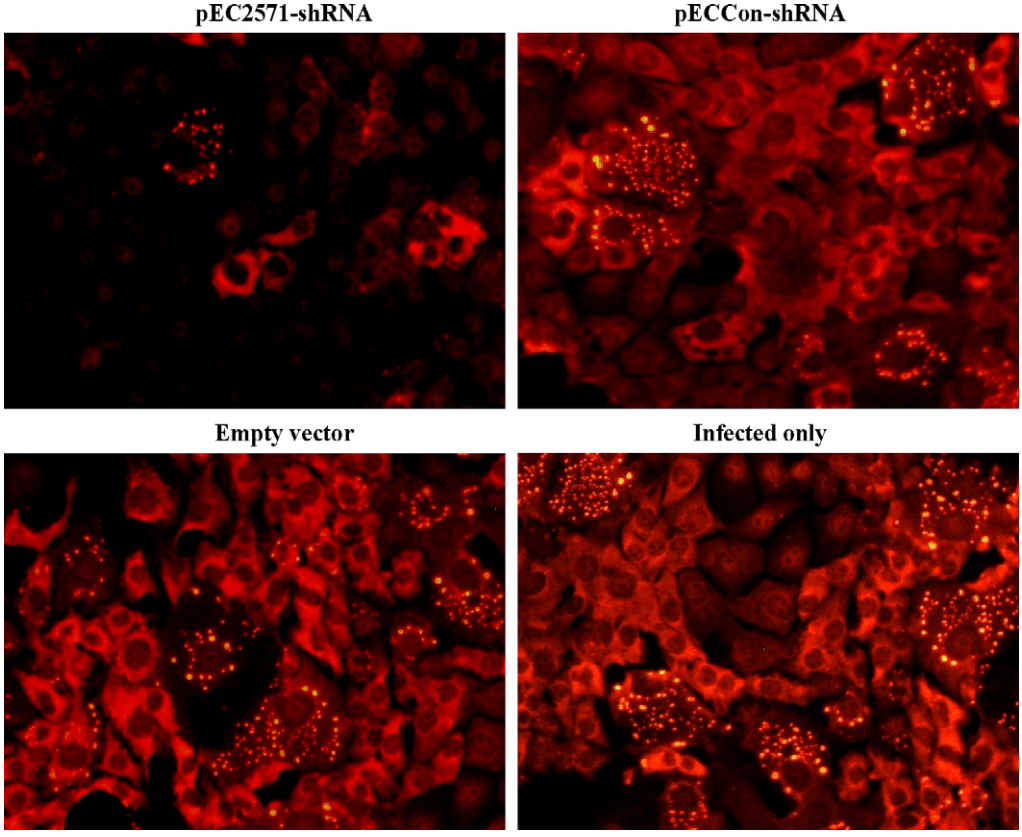

Fig. 5. Indirect immunofluorescence detection of IBDV in Vero cells previously transfected with or without shRNA-expressing vectors. The cells were transfected with shRNA-expressing vectors (1 μg each) in a 12-well plate. Sixty hours post-infection (MOI = 0.01), the cells were reacted against the anti-VP1 monoclonal antibody (MAb) and then incubated with the TRITC-conjugated secondary antibody. The second antibody was diluted with PBS containing Evan's blue. The bright red color indicated the presence of the VP1 protein-positive Vero cells. The fluorescence microscopy data was processed using the Adobe Photoshop software.

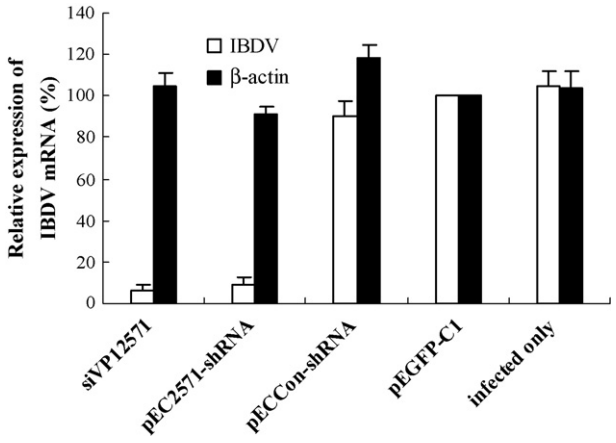

Fig. 6. VP1-specific siRNA inhibits the accumulation of VP1 mRNA. Vero cells were transfected with variant shRNA-expressing vectors and then infected with IBDV at an MOI of 0.01. Sixty hours post-infection, total RNA was extracted and subjected to real-time PCR analysis. The β-actin mRNA served as an internal reference. Black bars represent the mRNA level of β-actin; white bars represent the mRNA level of IBDV VP1. The data shown represents the mean value for three separate experiments; standard deviations indicated by error bars.

NAs are delivered into the mammalian cells in vitro. A minimal concentration range (nmol) of siRNA is particularly effective in this regard. As compared to the gene knockout strategies, RNAi is a shortcut that provides information regarding gene function rapidly and easily and is also inexpensive. The RNAi approach has been used for inhibiting the expression of viral proteins and reducing viral titers. Currently this technique is widely used in the research of animal viral diseases (such as FMDV, AIV, ALV, IBV, MDV, PRRSV, and etc.) and provides a new way for prevention and treatment of animal viral diseases (Chen et al., 2005, 2007; Huang et al., 2006; Kong and Cheng, 2005; Levy et al., 2005; Liu et al., 2005a,b). Thus, in this study, we used siRNA-expressing plasmids to inhibit IBDV replication.

IBDV VP1 is the viral RdRp that is associated with the transcription and replication of viral dsRNA (Kibenge et al., 1997a; Morgan et al., 1988). It exists either as an isolated monomer or in combination with the 5′ end of the A and B segments of the dsRNA genome for its cyclizing (Mirriam et al., 2002). Moreover, VP1 catalyzes the guanosine monophosphate reaction and initiates dsRNA synthesis (Kibenge and Dhama, 1997b; Macreadie and Azad, 1993; Spies and Muller, 1990). VP1 and VP3 form a complex that is involved in the replication and packaging of the viral genome (Tacken et al., 2000); furthermore, complex formation facilitates the generation of a complete viral particle (Lombardo et al., 1999). Since VP1 is responsible for viral genome replication and RNA synthesis, in this study, we selected the VP1 gene as the target for designing siRNA. The VPA revealed that the inhibition rate of the 3 selected siRNAs (siVP1^{618}, siVP1^{1115}, and siVP1^{2571}) on IBDV replication was greater than 80% among these, the inhibitory effect of siVP1^{2571} was the highest at 93.3%. These results demonstrated that siVP1^{2571} site is the most effective RNAi target site.

The virus plaque assay showed that the virus titer of the Vero cells transfected with the control siRNA^{Con} were equivalent to those of the non-transfected cells, indicating that under the transfection conditions of this study, the transfection reagent had no obvious impact on the proliferative ability of IBDV. However, in the Vero cells transfected with siVP1^{618}, siVP1^{1115}, and siVP1^{2571}, viral proliferation was effectively inhibited by the introduced siRNA, illustrating that the siRNA designed in this study was specific.

In 2002, Donze et al. reported that T7 RNA polymerase was used to synthesize dsRNA by in vitro transcription (Donze and Picard, 2002). Currently, this method is widely applied (Bantounas et al., 2004; Cozzi et al., 2004; Dowler et al., 2006; Hui et al., 2004; Paula et al., 2007; Sohail et al., 2003). This method permits researchers to rapidly obtain siRNA, and it is suitable for screening highly effective siRNA sequences (Dudek and Picard, 2004). The cost of in vitro transcription is relatively less than that of chemical synthesis. Moreover, via in vitro transcription, the resultant siRNA is less toxic, has good stability, and high transfection efficiency. Only 1/10 the amount of the chemically synthesized siRNA is required to achieve an identical effect (Caplen et al., 2001). Therefore, in this study, we selected this method for screening for highly effective siRNA sequences. Nevertheless, the disadvantage of this method is that the scale of the experiment is restricted and is unsuitable for both large-scale synthesis of specific siRNAs and long-term research.

In order to reduce research costs, expression vectors are usually constructed to inhibit viral proliferation after the transfection of cells with expressed siRNA (Adelman et al., 2001; Chen et al., 2004; Kahana et al., 2004; Yokota et al., 2003). In this study, siRNA efficacy was screened for via in vitro transcription, and the RNA polymerase III promoter-mouse U6 promoter was used to construct the siRNA expression vector (Elbashir et al., 2002; Lee et al., 2002). The plasmid pEGFP-C1 encodes for enhanced green fluorescence protein (EGFP). This gene was retained during vector construction to enable assessment of the transfection efficiency by observing EGFP expression in transfected cells.

After the Vero cells were transfected with the constructed shRNA expression vector pEC2571-shRNA, we observed that the level of inhibition with the shRNA expressing vector is equivalent or a little lower than the inhibition produced by siVP1^{2571} synthesized in vitro, as determined by plaque assay and real time PCR. siRNA effects are dependent on sequence (Holen et al., 2002; Harborth et al., 2003). siRNA and shRNA in this research targeted the same sites of VP1 gene of IBDV. Presumably, this is because transfection of a large vector is less efficient than for small oligonucleotides and because in vitro transcription synthesized oligomers begin to act immediately, whereas the hairpin oligonucleotides are gradually synthesized within the cell (Dong et al., 2004). As compared to that after the pECCon-shRNA transfection of the Vero cells, no obvious inhibitory effect was observed on IBDV replication (Figs. 2–6). The results indicated that the shRNA generated by U6 promoter transcription in vitro was specific. Real-time PCR revealed that the VP1 mRNA level in the cells treated with pEN-shRNA and pEGFP-C1 empty plasmids did not significantly decrease as compared to that in the non-transfected controls, demonstrating that the decrease in the VP1 mRNA level was specific but not caused by low transfection efficiency and liposome toxicity.

In conclusion, this study demonstrates that DNA vector-based RNAi effectively inhibits IBDV replication in vitro. Further study is required to determine whether such treatment offers protection against IBDV infection in vivo. However, this work represents a significant advance, potentially facilitating new experimental approaches for the analysis of both viral and cellular gene functions in the context of IBDV infection.

Acknowledgments

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References

Adelman, Z.N., Blair, C.D., Carlson, J.O., Beaty, B.J., Olson, K.E., 2001. Sindbis virus-induced silencing of dengue viruses in mosquitoes. Insect. Mol. Biol. 10, 265–273.

Bantounas, I., Phylactou, L.A., Uney, J.B., 2004. RNA interference and the use of small interfering RNA to study gene function in mammalian systems. J. Mol. Endocrinol. 33, 545–557.

Brown, S.J., Mahaffey, J.P., Lorenzen, M.D., Denell, R.E., Mahaffey, J.W., 1999. Using RNAi to investigate orthologous homeotic gene function during the development of distantly related insects. Evol. Dev. 1, 11–15.

Brummelkamp, T.R., Bernards, R., Agami, R., 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550–553.

Burkhardt, E., Muller, H., 1987. Susceptibility of chicken blood lymphoblasts and monocytes to infectious bursal disease virus (IBDV). Arch. Virol. 94, 297–303.

Caplen, N.J., Parrish, S., Imani, F., Fire, A., Morgan, R.A., 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc. Natl. Acad. Sci. USA 98, 9742–9747.

Chen, J., Wall, N.R., Kocher, K., Duclos, N., Fabbro, D., Neuberg, D., Griffin, J.D., Shi, Y., Gilliland, D.G., 2004. Stable expression of small interfering RNA sensitizes TEL-PDGFβR to inhibition with imatinib or rapamycin. J. Clin. Invest. 113, 1784–1791.

Chen, M., Granger, A.J., VanBrocklin, M.W., Payne, W.S., Hunt, H., Zhang, H., Jerry, B.D., Sheri, L.H., 2007. Inhibition of avian leukosis virus replication by vector-based RNA interference. Virology 365, 464–472.

Chen, W., Yan, W., Zheng, Z., 2005. Response to Bayry and Tough: Is RNAi useful for controlling foot-and-mouth disease outbreaks. Trends Immunol. 26, 239–241.

Chettle, N., Stuart, J.C., Wyeth, P.J., 1989. Outbreak of virulent infectious bursal disease in East Anglia. Vet. Rec. 125, 271–272.

Cottrell, T.R., Doering, T.L., 2003. Silence of the strands: RNA interference in eukaryotic pathogens. Trends Microbiol. 11, 37–43.

Cozzi, A., Corsi, B., Levi, S., Santambrogio, P., Biasiotto, G., Arosio, P., 2004. Analysis of the biologic functions of H- and L-ferritins in HeLa cells by transfection with siRNAs and cDNAs: evidence for a proliferative role of L-ferritin. Blood 103, 2377–2383.

Dobos, P., Hill, B.J., Hallett, R., Kells, D.T., Becht, H., Teninges, D., 1979. Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. J. Virol. 32, 593–605.

Dong, X., Hyunmin, K., Michael, F., Juliano, R.L., 2004. Strategies for Inhibition of MDR1 Gene Expression. Mol. Pharmacol. 66, 268–275.

Donze, O., Picard, D., 2002. RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. Nucleic Acids Res. 30, e46.

Dowler, T., Bergeron, D., Tedeschi, A.L., Paquet, L., Ferrari, N., Damha, M.J., 2006. Improvements in siRNA properties mediated by 2′-deoxy-2′-fluoro-beta-D-arabinonucleic acid (FANA). Nucleic Acids Res. 34, 1669–1675.

Downward, J., 2004. RNA interference. BMJ 328, 1245–1248.

Dudek, P., Picard, D., 2004. T7 RNAi Oligo Designer. Nucleic Acids Res. 32, 121–123.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494–498.

Elbashir, S.M., Harborth, J., Weber, K., Tuschl, T., 2002. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 26, 199–213.

Eterredossi, N., Gauthier, C., Reda, I., Comte, S., Rivallan, G., Toquin, D., Boisseson, C., Lamande, J., Jestin, V., Morin, Y., Cazaban, C., Borne, P., 2004. Extensive antigenic changes in an atypical isolate of very virulent infectious bursal disease virus and experimental clinical control of this virus with an antigenically classical live vaccine. Avian Pathol. 33, 423–431.

Harborth, J., Elbashir, S.M., Vandenburgh, K., Manninga, H., Scaringe, S.A., Weber, K., Tuschl, T., 2003. Sequence, chemistry, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. Antisense Nucleic Acid Drug Dev. 13, 83–105.

Holen, T., Amarzguioui, M., Wiiger, M.T., Babaie, E., Prydz, H., 2002. Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. Nucleic Acids Res. 30, 1757–1766.

Huang, J., Jiang, P., Li, Y., Xu, J., Jiang, W., Wang, X., 2006. Inhibition of porcine reproductive and respiratory syndrome virus replication by short hairpin RNA in MARC-145 cells. Vet. Microbiol. 115, 302–310.

Hudson, P.J., Mckern, N.M., Power, B.E., Azad, A.A., 1986. Genomic structure of the large RNA segment of infectious bursal virus. Nucleic Acides Res. 14, 5001–5012.

Hui, E.K.W., Yap, E.M., An, D.S., Chen, I.S.Y., Nayak, D.P., 2004. Inhibition of influenza virus matrix (M1) protein expression and virus replication by U6 promoter-driven and lentivirus-mediated delivery of siRNA. J. Gen. Virol. 85, 1877–1884.

Kahana, R., Kuznetzova, L., Rogel, A., Shemesh, M., Hai, D., Yadin, H., Stram, Y., 2004. Inhibition of foot-and-mouth disease virus replication by small interfering RNA. J. Gen. Virol. 85, 3213–3217.

Kibenge, F.S., Cleghorn, J.R., Qian, B., 1997a. Infectious bursal disease virus polyprotein processing does not involve cellular proteases. Arch. Virol. 142, 2401–2419.

Kibenge, F.S., Dhama, V., 1997b. Evidence that viriorn associated VP1 of avibirnaviruses contains viral RNA sequence. Arch. Virol. 142, 1227–1236.

Kong, L.H., Cheng, B.Z., 2005. Construction of influenza virus siRNA expression vectors and their inhibitory effects on multiplication of influenza virus. Avian Dis. 49, 562–573.

Lee, N.S., Dohjima, T., Bauer, G., Li, H., Li, M.J., Ehsani, A., Salvaterra, P., Rossi, J., 2002. Expression of small interfering RNA stargeted against HIV-1 rev transcripts in human cell. Nat. Biotechnol. 20, 500–505.

Levy, A.M., Gilad, O., Xia, L., Izumiya, Y., Choi, J., Tsalenko, A., Yakhini, Z., Witter, R., Lee, L., Cardona, C.J., Kung, H.J., 2005. Marek's disease virus Meq transforms chicken cells via the v-Jun transcriptional cascade: A converging transforming pathway for avian oncoviruses. Proc. Natl. Acad. Sci. USA 102, 14831–14836.

Liu, H.L., Lu, C.P., Zhu, W.Y., 2005a. RNA Interference Against Avian Infectious Bronchitis Virus. Virol. Sin. 20, 272–276.

Liu, M., Chen, W., Ni, Z., 2005b. Cross-inhibition to heterologous foot-and-mouth disease virus infection induced by RNA interference targeting the conserved regions of viral genome. Virology 336, 51–59.

Lombardo, E., Maraver, A., Caston, J.R., Rivera, J., Fernández-Arias, A., Serrano, A., Carrascosa, J.L., Rodriguez, J.F., 1999. VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles. J. Virol. 73, 6973–6983.

Macreadie, I.G., Azad, A.A., 1993. Expression and RNA dependent RNA polymerase activity of birnavirue VP1 protein in bacteria and yeast. Biochem. Mol. Biol. Int. 30, 1169–1178.

Mirriam, G.J.T., Peeters, B.P.H., Thomas, A.A.M., Rottier, P.J.M., Boot, H.J., 2002. Infectious Bursal Disease Virus Capsid Protein VP3 Interacts both with VP1, the RNA-Dependent RNA Polymerase, and with Viral Double-Stranded RNA. J. Virol. 76, 11301–11311.

Miyagishi, M., Sumimoto, H., Miyoshi, H., Kawakami, Y., Taira, K., 2004. Optimization of an siRNA-expression system with an improved hairpin and its significant suppressive effects in mammalian cells. J. Gene. Med. 6, 715–723.

Miyagishi, M., Taira, K., 2002. U6 promotor-driven siRNAs with four uridine 3′-veerhangs efficiently suppress targeted gene expression in mammalian cells. Nat. Biotechol. 20, 497–505.

Morgan, M.M., Macreadie, I.G., Harley, V.R., Hudson, P.J., Azad, A.A., 1988. Sequence of the small double-stranded RNA genomic segment of infectious bursal disease virus and its deduced 90 kDa product. Virology 163, 240–242.

Müller, H., 1986. Replication of infectious bursal disease virus in lymphoid cells. Arch. Virol. 87, 191–203.

Paddison, P.J., Caudy, A.A., Sachidanandam, R., Hannon, G.J., 2004. Short hairpin activated gene silencing in mammalian cells. Methods Mol. Biol. 265, 85–100.

Paul, C.P., Good, P.D., Winer, I., Engelke, D.R., 2002. Effective expression of small interfering RNA in human cells. Nat. Biotechnol. 20, 505–508.

Paula, D.D., Bentley, M.V.L.B., Mahato, R.I., 2007. Hydrophobization and bioconjugation for enhanced siRNA delivery and targeting. RNA 13, 431–456.

Pinkenburg, O., Platz, J., Beisswenger, C., Vogelmeier, C., Bals, R., 2004. Inhibition of NF-kappaB mediated inflammation by siRNA expressed by recombinant adeno-associated virus. J. Virol. Methods 120, 119–122.

Rubinson, D.A., Dillon, C.P., Kwiatkowski, A.V., Sievers, C., Yang, L., Kopinja, J., Rooney, D.L., Ihrig, M.M., McManus, M.T., Gertler, F.B., Scott, M.L., Van Parijs, L., 2003. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat. Genet. 33, 401–406.

Sivanandan, V., Maheswaran, S.K., 1980. Immune profile of infectious bursal disease: I Effect of bursal disease virus on peripheral blood T and B lymphocytes of chickens. Avian Dis. 24, 715–725.

Sohail, M., Doran, G., Riedemann, J., Macaulay, Val., Southern, E.M., 2003. A simple and cost-effective method for producing small interfering RNAs with high efficacy. Nucleic Acids Res. 31, e38.

Spies, U., Muller, H., 1990. Demonstration of enzyme activities required for cap structure formation in infectious bursal disease virus, a member of the birnavirus group. J. Gen. Virol. 71, 977–981.

Sui, G.C., Soohoo, C., Affar, E.B., Gay, F., Shi, Y.J., Forrester, W.C., Shi, Y., 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. USA 99, 5515–5520.

Tacken, M.G., Rottier, P.J., Gielkens, A.L., Peeters, B.P., 2000. Interactions in vivo between the proteins of infectious bursal disease virus: capsid protein VP3 interacts with the RNA-dependent RNA polymerase VP1. J. Gen. Virol. 81, 209–218.

Wang, X.M., Zeng, X.W., Gao, H.L., Fu, C.Y., 2004. Changes in VP2 gene during the attenuation of very virulent infectious bursal disease virus strain Gx isolated in china. Avian Dis. 48, 77–83.

Yokota, T., Sakamoto, N., Enomoto, N., Tanabe, Y., Miyagishi, M., Maekawa, S., Yi, L., Kurosaki, M., Taira, K., Watanabe, M., Mizusawa, H., 2003. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep. 4, 1–7.

Yu, J.Y., DeRuiter, S.L., Turner, D.L., 2002. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cell. Proc. Natl. Acad. Sci. USA 99, 6047–6052.

Zhao, L.J., Jian, H., Zhu, H., 2003. Specific gene inhibition by adenovirus-mediated expression of small interfering RNA. Gene 316, 137–141.