Available online at www.sciencedirect.com
ScienceDirect
Antiviral Research 74 (2007) 83–91
Antiviral Research
www.elsevier.com/locate/antiviral

Interference of porcine reproductive and respiratory syndrome virus replication on MARC-145 cells using DNA-based short interfering RNAs

Yun-xia He, Rong-hong Hua, Yan-jun Zhou, Hua-ji Qiu, Guang-zhi Tong *

National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 427 Maduan Street, Harbin 150001, PR China

Received 17 January 2006; accepted 18 April 2006

Abstract

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease in swine-producing areas of the world. Many vaccine strategies developed to control the disease are not yet completely successful. The objective of this study was to determine if RNA interference (RNAi) could be utilized to inhibit PRRSV replication on MARC-145 cells. Four short interfering RNA (siRNA) sequences (N95, N179, N218 and N294) directed against a well-conserved region of PRRSV genome ORF7 gene were selected. Sense and antisense siRNA encode sequences separated by a hairpin loop sequence were designed as short hairpin RNA (shRNA) expression cassettes driven by mouse U6 promoter. Using a polymerase chain reaction (PCR)-based approach, shRNAs were generated from shRNA expression cassettes. The PCR products were cloned into pEGFP-N1 vector and shRNA expression vectors were constructed. When MARC-145 cells were transfected with shRNA expression vectors and then infected with PRRSV, N179 was found to be the most effective inhibition site in decreasing cytopathic effect (CPE) induced by PRRSV. Western blot, indirect immunofluorescence and fluorescence quantitative PCR (FQ-PCR) confirmed that the expression of ORF7 was reduced both at protein and RNA levels comparing to controls. The results presented here indicated that DNA-based siRNA could effectively inhibit the replication of PRRS virus (approximately 681-fold reduction of viral titers) on MARC-145 cells.

Keywords: PRRSV; RNAi; shRNA; siRNA

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is characterized by respiratory disorders in young pigs and reproductive failure in sows. It is widespread in most major pig-producing areas throughout the world and is one of the most important causes of economic loss to the swine industry (Collins et al., 1992; Pejsak and Markowska-Daniel, 1997; Wensvoort, 1993). This disease is due to the infection caused by porcine reproductive and respiratory syndrome virus (PRRSV), which is classified as a member of the order of *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* (Cavanagh, 1997; Meulenberg et al., 1993b). PRRSV has an approximately 15 kb single-stranded polyadenylated RNA genome that encodes at least nine partially overlapping open reading frames (ORFs). ORFs 1a and 1b occupy more than two-thirds of the genome and encode the viral RNA polymerase. ORFs 2–7 are located at the 3′-end of the genome and produced as a 3′-coterminal nested set of subgenomic mRNA during replication and encode the structural proteins (Meulenberg et al., 1993a; Snijder et al., 1999; Wu et al., 2001). ORF7 encodes the nucleocapsid protein N which constitutes about 20–40% of the total protein content of the virion and is essential during the assembly and disassembly of virion (Bautista et al., 1996; Mardassi et al., 1994; Nelson et al., 1993; Verheije et al., 2001).

RNA interference (RNAi) is a conserved gene silencing mechanism that recognizes double-stranded RNA as a signal to trigger the sequence-specific degradation of homologous mRNA (Sharp and Zamore, 2000). At the heart of the pathway are two ribonuclease machines. The ribonuclease III enzyme Dicer initiates the RNAi pathway by generating the active short interfering RNA trigger. Silencing is effected by the RNA-induced silencing complex and its RNase H core enzyme Argonaute (Elbashir et al., 2001; Hammond, 2005). In mammalian cells, RNAi can be triggered by chemically or enzymatically synthesized 21–25 nt long RNA duplexes (Elbashir et al., 2001;

* Corresponding author. Tel.: +86 451 82734181; fax: +86 451 82734181.
E-mail address: gztong@hvri.ac.cn (G.-z. Tong).

0166-3542/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2006.04.013

Sohail et al., 2003). As 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 (Brummelkamp et al., 2002b; Pinkenburg et al., 2004; Rubinson et al., 2003; Zhao et al., 2003). These vectors use RNA polymerase III promoters to direct the synthesis of short hairpin RNA (shRNA) molecules, which are processed intracellularly into siRNA-like molecules. Vector-produced shRNA molecules are as effective as siRNAs that are generated in vitro in inhibiting gene expression. Hairpin expression vectors have been used to induce gene silencing in a numbers of studies on viruses (Brummelkamp et al., 2002a; Brummelkamp et al., 2002b; McCaffrey et al., 2003; Miyagishi and Taira, 2002; Paddison et al., 2004; Paul et al., 2002). In the interest of exploiting improved methods to control PRRS, we have hypothesized that RNAi may provide effective protection against PRRSV. Here we apply a vector-based siRNA technology to inhibit the replication of PRRSV on MARC-145 cells.

2. Materials and methods

2.1. Selection of target sequences and construction of shRNA expression cassette

The plasmid pBS-U6 (a gift of Dr. Guangchao Sui, Harvard Medical School) was used as a template for polymerase chain reaction (PCR) amplification of the mouse U6 promoter (−315 to +1) (Sui et al., 2002). Four 19 nt siRNAs (N95, N179, N218 and N294) from four different regions of the ORF7 gene (GenBank accession number AY032626) were selected using the siRNA "Target Finder and Design Tool" available at http://www.ambion.com/ (Fig. 1A). Specificity of these sequences was verified by BLAST search. Additionally, a non-specific siRNA sequence was designed to examine inhibitory specificity. Using a PCR-based strategy, shRNA expression cassettes were designed. In this strategy, a universal forward primer that is complementary to the 5′-end of the mouse U6 promoter is used. The reverse primers harbor 19 nt sequence complementary to the 3′-end of the promoter, the sense and antisense sequence of siRNA, a loop sequence, five adenosines and the restriction enzyme sites sequence (Fig. 1B). The resulting PCR products are shRNA expression cassettes under the control of mouse U6 promoter. To facilitate optional cloning of the PCR expression cassette into vectors, the *Eco*O109I restriction site sequences were designed in both the forward and reverse primers. The 9 nt loop sequence (Brummelkamp et al., 2002b) was common to all of the hairpins. All primers were synthesized by Sangon (Shanghai, China). The resulting PCR products were digested with *Eco*O109I and cloned into the pEGFP-N1 vector (BD Biosciences Clontech, Palo Alto, USA). The recombinant plasmids, pEN95-shRNA, pEN179-shRNA, pEN218-shRNA, pEN294-shRNA and nonspecific control pEC-shRNA, were sequenced for verification. Plasmids used for transfection were purified with the Wizard Purefection™ Plasmid DNA Purification System (Promega, Madison, WI, USA) and quantified by Eppendorf Biophotometer. The Forward primer was: 5′-AGTGAGGTCCTGATCCGACGCCGCCATCTCTA-3′. Reverse primers are listed in Table 1.

2.2. Cell culture, transfection and virus infection

The PRRSV permissive cell line, MARC-145, a monkey kidney cell line, maintained in Harbin Veterinary Research Institute, China, was grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Inc., San Diego, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 170 mM penicillin and 40 mM streptomycin. The cultures were maintained in a 5% CO_2 humidified incubator at 37 °C. The cells were trypsinized and plated in 12-well plates at 10^5 cells per well 16 h before transfection. For transfection, we used 1 μg of each plasmid in 100 μl of OPTI-MEM medium (Gibco BRL, Gaithersburg, MD) mixed with lipofectamine 2000 (Invitrogen, Inc.) according to the manufacturer's instructions and all 12-well plates were with

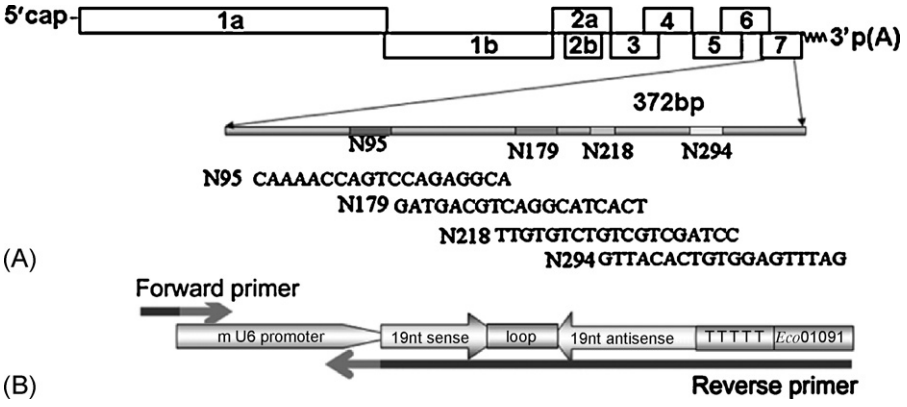

Fig. 1. Target small interference RNAs (siRNAs) and polymerase chain reaction (PCR)-based strategy for short hairpin RNA (shRNA) expression cassettes. (A) Genomic structure of porcine reproductive and respiratory syndrome virus (PRRSV) and position of target siRNAs: N95, N179, N218 and N294. (B) PCR-based strategy for shRNA expression cassettes. For forward primer arrow, grey section represents sequences according to mouse U6 promoter, black section represents *Eco*O109I site. For reverse primer arrow, grey section represents sequences complement to mouse U6 promoter and black section represents hairpin and five adenosines and *Eco*O109I site.

Table 1
Sequences of siRNA and reverse primers

Downstream primers	siRNA sequence and reverse primers
U6P2-N95	5′-GACAG′GACCTAAAAA***CAAAACCAGTCCAGAGGCA*** tctcttgaa**TGCCTCTGGACTGGTTTTG**AAACAAGGCTTTTCTCCAA-3′
U6P2-N179	5′-GACAG′GACCTAAAAA***GATGACGTCAGGCATCACT*** tctcttgaa**AGTGATGCCTGACGTCATC**AAACAAGGCTTTTCTCCAA-3′
U6P2-N218	5′-GACAG′GACCTAAAAA***TTGTGTCTGTCGTCGATCC*** tctcttgaa**GGATCGACGACAGACACAA**AAACAAGGCTTTTCTCCAA-3′
U6P2-N294	5′-GACAG′GACCTAAAAA***GTTACACTGTGGAGTTTAG*** tctcttgaa**CTAAACTCCACAGTGTAAC**AAACAAGGCTTTTCTCCAA-3′
$U6P_2$-control	5′-GACAG′GACCTAAAAA***GACCTTGATCCGTCTTACC*** tctcttgaa**GGTAAGACGGATCAAGGTC**AAACAAGGCTTTTCTCCAA-3′

*Eco*O109I site are underlined. Sense and antisense siRNA sequences are bolded (sense strands are italicized). The lower-case characters are loop sequences.

1 ml medium per well. This concentration was determined to be optimal in preliminary experimentation. Specificity of the inhibition was confirmed by transfecting the cultures with pEGFP-N1 empty vector and the nonspecific vector pEC-shRNA. Four hours post-transfection, the cells were infected with PRRSV CH-1a isolate (Guo et al., 1996) at a multiplicity of infection (MOI) of 0.01. The cultures were then incubated at 37 °C, 5% 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 before Western blot and fluorescence quantitative PCR (FQ-PCR) analysis. Culture supernatants were collected for virus titration.

2.3. Virus titration

MARC-145 cells were seeded into 96-well microtitre plates 1 day before infection. Supernatant samples obtained above were 10-fold serially diluted and added to wells, 100 μl per well in triplicate. Five days after infection, the 50% cell culture infectious dose ($CCID_{50}$) was calculated by the Spearman–Karber method (Kaerber, 1931).

2.4. Indirect immunofluorescece

Sixty hours after infection, cells grown in 12-well plates were trypsinized. Following three washes in PBS (137 mM NaCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4), cells were pelleted by low-speed centrifugation at 1500 rpm for 1 min to remove the supernatant. Cells were resuspended in PBS and added drop-wise onto slides and fixed with cool acetone for 20 min. After washing for three times with PBS, the fixed samples were incubated with monoclonal antibody (MAb) directed against the N protein (Zhou et al., 2005) of PRRSV CH-1a isolate for 1 h at 37 °C. Cells were washed three times and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (H + L) (Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:100, for 1 h at 37 °C. After three PBS washes and one additional wash with deionized H_2O, slides were mounted with coverslips and analyzed by fluorescence microscopy (Leica, HC).

2.5. Reverse transcription and FQ-PCR analysis

The sequences of Taq-Man probes and primers are listed in Table 2 (synthesized by Takara, Dalian, China). MARC-145 cells were transfected with or without shRNA expression vectors. Four hours later, cultures were infected with PRRSV. Sixty hours post-infection, the culture medium was removed and the cells were harvested for total RNA isolation using the RNeasy mini kit (Qiagen Inc., Valencia, CA, USA). Small amounts of contamination DNA were removed with the RNase-Free DNase Set (Qiagen Inc.) according to the manufacturer's recommendations. Reverse transcription was carried out according to the Takara reverse transcription reaction protocol in a volume of 30 μl at 42 °C for 1 h. One microliter of reverse transcription reaction mixture was used for FQ-PCR by using gene-specific primers, probes and TaqMan universal PCR master mixture (Takara). 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 at 95 °C for 15 s and 60 °C for 45 s. The target mRNAs in samples were quantified by comparison with a standard curve derived from known amounts plasmids of target gene. Relative amount of interesting mRNA were normalized to β-actin mRNA. Amplification and detection of samples were

Table 2
Taqman primers and probes

Gene name	Sequence
β-Actin	
Actin-F	5′-TGACTGACTACCTCATGAAGATCC-3′
Actin-R	5′-TCTCCTTAATGTCACGCACGATT-3′
Actin-Taq Man	5′-(FAM)-CGGCTACAGCTTCACCACCACGGC-(Eclipse)-3′
ORF7	
ORF7-F	5′-CCTCTAGCGACCGAAGATGAC-3′
ORF7-R	5′-CCCTGGTTAAAGGCAGTCTGG-3′
ORF7-Taq Man	5′-(ROX)-CGACGACAGACACAATTGCCGCTCA-(Eclipse)-3′
GFP	
GFP-F	5′-AAGATCCGCCACAACATCGAG-3′
GFP-R	5′-GGACTGGGTGCTCAGGTAGT-3′
GFP-Taq Man	5′-(ROX)-TGCAGCTCGCCGACCACTACCAG-(Eclipse)-3′

performed with the Rotor-gene 3000 detection system (Gene Company Limited).

2.6. Western blotting

MARC-145 cells transfected with shRNA expression vectors were collected and lysed in 2× SDS sample buffer. Proteins were analyzed by 12% sodium dodecyl–sulfate polyacrylamide gel electropheresis (SDS–PAGE) and transferred onto nitrocellulose membrane, blocked by 10% skim milk, and probed with anti-N protein MAb. To normalize protein loading, the membranes were simultaneously incubated with anti-GAPDH MAb (Sigma-Aldrich). As secondary antibody, a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (Sigma-Aldrich) was used at a dilution of 1:5000. Immunoblots were examined using supersignal west pico chemiluminescence substrate according to the manufacturer's instruction (Pierce, Rockford, IL, USA) and filter memberanes were exposed to Kodak X-OMAT X-ray film.

3. Results

3.1. Inhibition of CPE

To investigate whether RNAi could protect MARC-145 cells from cytopathic effect (CPE) induced by PRRSV, MARC-145 cells were transfected by plasmids expressing N protein-targeted shRNAs (pEN95-shRNA, pEN179-shRNA, pEN218-shRNA and pEN294-shRNA), respectively. As a control for nonspecific inhibition, pEGFP-N1 empty vector and nonspecific shRNA expression vector pEC-shRNA were transfected in parallel. To determine transfection efficiency, we monitored the GFP fluorescence intensity of transfected cells on an inverted fluorescent microscope (approximately 40%, data not shown). Four hours post-transfection, the cultures were infected with PRRSV. CPE were observed daily. Sixty hours later, we found that cells pre-transfected with pEN179-shRNA manifested overtly less CPE. Other shRNA-treated cells (A, C, D and E) and the empty vector control (F) demonstrated the same typical PRRSV-induced CPE as cells infected only with virus (G), which became pycnotic and detached from the monolayer, as shown in Fig. 2.

3.2. Indirect immunofluorescence assay

To investigate the effect of shRNA-expressing vectors in inhibiting the expression of N protein, indirect immunofluorescence assay was performed with anti-N protein MAb at 60 h post-infection. Indeed, overtly less fluorescing cells were seen in the monolayers pretreated with pEN179-shRNA than in the other monolayers. In contrast, the infected cells in wells pre-transfected with pEN95-shRNA, pEN218-shRNA and pEN294-shRNA had equivalent or slightly less N protein-positive cells compared to that of controls (Fig. 3). In addition, no fluorescing cells were seen in the empty vector transfected MARC-145 cells with anti-N protein-specific MAb (data not shown).

3.3. Western blotting analysis

The N protein is a small protein with an approximate molecular mass of 15 kDa. To further analyze the suppression effect of pEN179-shRNA on protein level, MARC-145 cells were transfected with 1 μg pEN179-shRNA and then infected with PRRSV at an MOI of 0.01. Sixty hours post-infection, the cells were collected to assay by Western blot. Only infected cells were shown to express the expected 15 kDa protein-N band by anti-

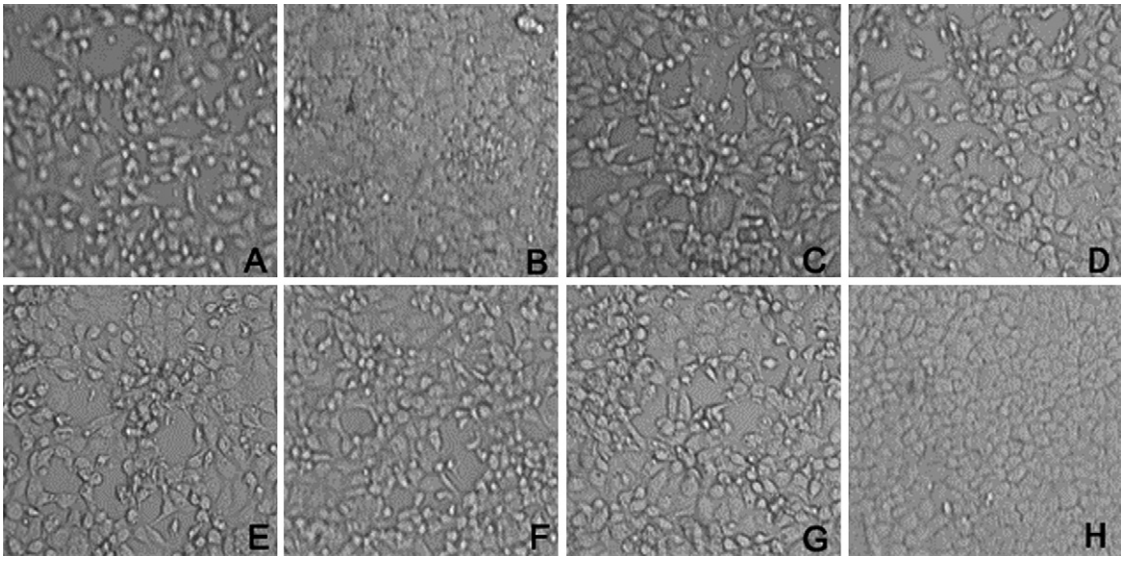

Fig. 2. Cytopathic effect (CPE) analysis of porcine reproductive and respiratory syndrome virus (PRRSV) on MARC-145 cells transfected with shRNA-expressing vectors (1 μg each). (A)–(F) representation of cells transfected with pEN95-shRNA, pEN179-shRNA, pEN218-shRNA, pEN294-shRNA, pEC-shRNA and empty vector (pEGFP-N1). (G) and (H) represented mock-transfected and untreated cells, respectively. Except (H), all cells were infected with PRRSV CH-1a isolate at a multiplicity of infection (MOI) of 0.01. Pictures were taken at 60 h post-infection with a Nikon E5400 camera mounted on an inverted microscope (Nikon TS100).

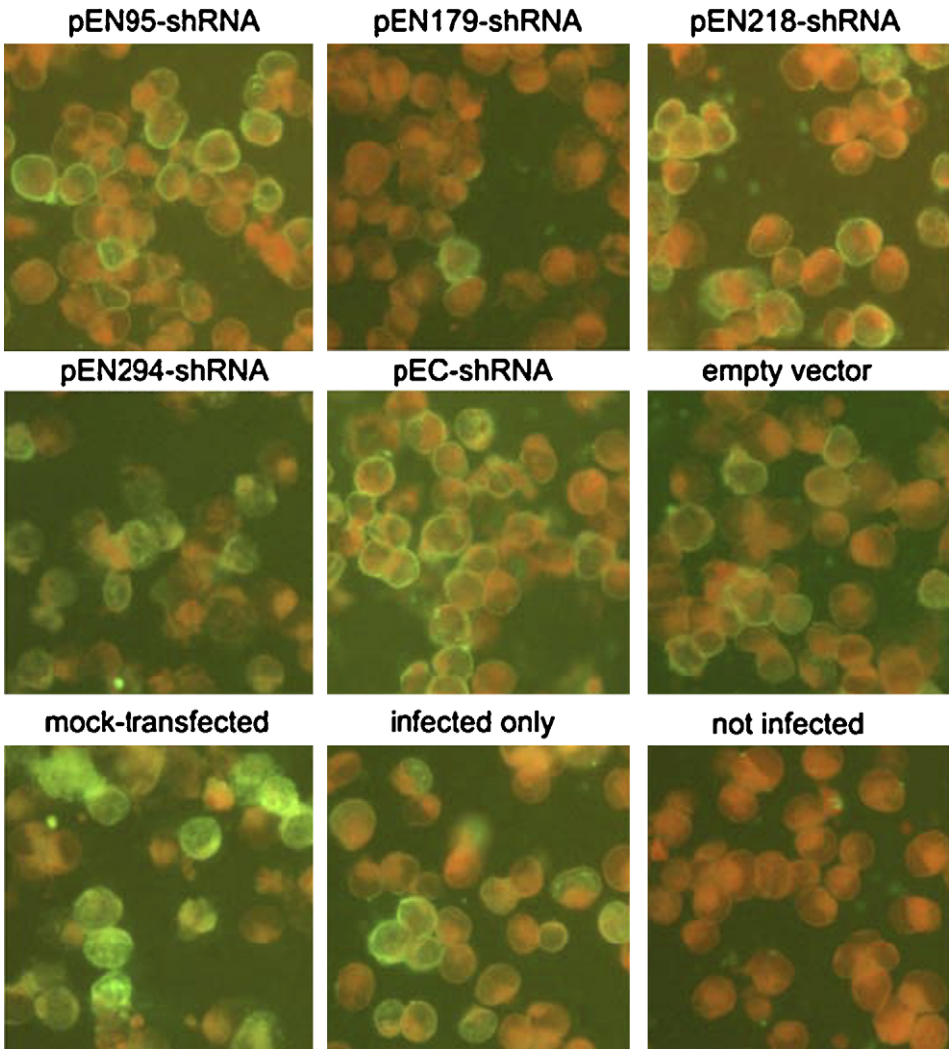

Fig. 3. Indirect immunofluorescence detection of porcine reproductive and respiratory syndrome virus (PRRSV) on MARC-145 cells previously transfected with or without short hairpin RNA (shRNA)-expressing vectors. Cells were transfected with shRNA-expressing vectors (1 μg each) in a 12-well plate. Sixty hours post-infection (MOI = 0.01), cells were reacted against anti-N monoclonal antibody (MAb) and then incubated with fluoresce in isothiocyanate (FITC) conjugated secondary antibody. The second antibody was diluted with PBS containing Evan's blue. The bright green color means N protein-positive MARC-145 cells. Dark yellow color means negative cells. Fluorescence microscopy data was processed using the Adobe Photoshop software.

N MAb. Lysates from cells transfected with the pEC-shRNA showed a similar pattern of reactivity. In contrast, in cells that were previously transfected with pEN179-shRNA, only a faint band could be seen. Normal cells showed no 15 kDa protein-N band (Fig. 4A). To investigate whether the inhibitory effect of pEN179-shRNA was dose-dependent, MARC-145 cells were transfected with 0.4, 0.8, 1.5 and 2.0 μg of this vector. Subsequently, the cells were infected with PRRSV and the whole cell lysates were examined by Western blot. The results showed that the silencing effect of pEN179-shRNA was dose-dependent (Fig. 4B).

3.4. Fluorescence quantitative PCR analysis

To measure the level of gene suppression accurately, FQ-PCR primers and Taqman probes directing to ORF7 were designed. We also designed probes and primers directed to EGFP (to monitor the equality of transfection efficiency) and β-actin sequence (serve as internal reference). When normalized for loading differences using the β-actin mRNA, the ORF7 message in the cells transfected with pEN95-shRNA, pEN179-shRNA, pEN218-shRNA and pEN294-shRNA were reduced by 49, 96, 42 and 51% (ORF7 message copies ratios of cells transfected with shRNA expression vectors/cells transfected with empty vector) (Fig. 5A). There was no significant inhibition in cells transfected with the empty vector and nonspecific shRNA expression vector pEC-shRNA. mRNA levels of EGFP (average EGFP mRNA levels in cells treated with pEN95-shRNA, pEN179-shRNA, pEN218-shRNA, pEN294-shRNA, pEC-shRNA and empty vector were 1.78E + 07, 1.21E + 07, 2.60E + 07, 8.15E + 06, 8.00E + 06, 2.34E + 07, respectively) or β-actin (average β-actin mRNA levels in cells treated with N95, N179, N218, N294, pEC-shRNA, empty vector, infected only and normal cells were 1.64E + 08, 1.91E + 08, 1.45E + 08,

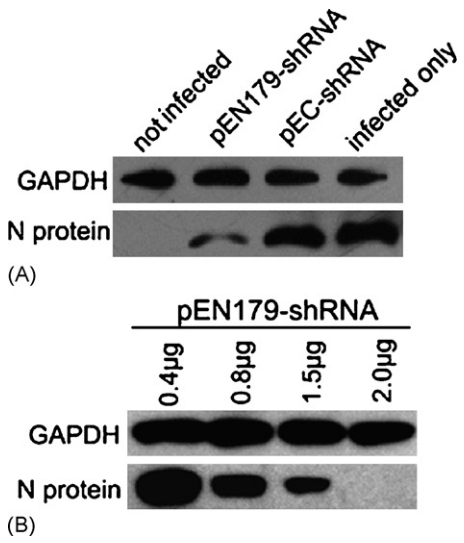

Fig. 4. Western blot analysis of gene suppression in protein level. Whole-cell extracts of shRNA-transfected cells were prepared 60 h post-infection (MOI = 0.01), separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Glyceraldehyde phosphate dehydrogenase (GAPDH) protein served as loading control. (A) Reduction of N protein levels by pEN179-shRNA (1 μg/ml). (B) Dose-dependent inhibitory effect in reduction of N protein levels by pEN179-shRNA. At here, 0.4, 0.8, 1.5 and 2.0 μg pEN179-shRNA were used in 12-well plate with 1 ml medium per well. Data are from a representative experiment and were processed using the Adobe Photoshop software.

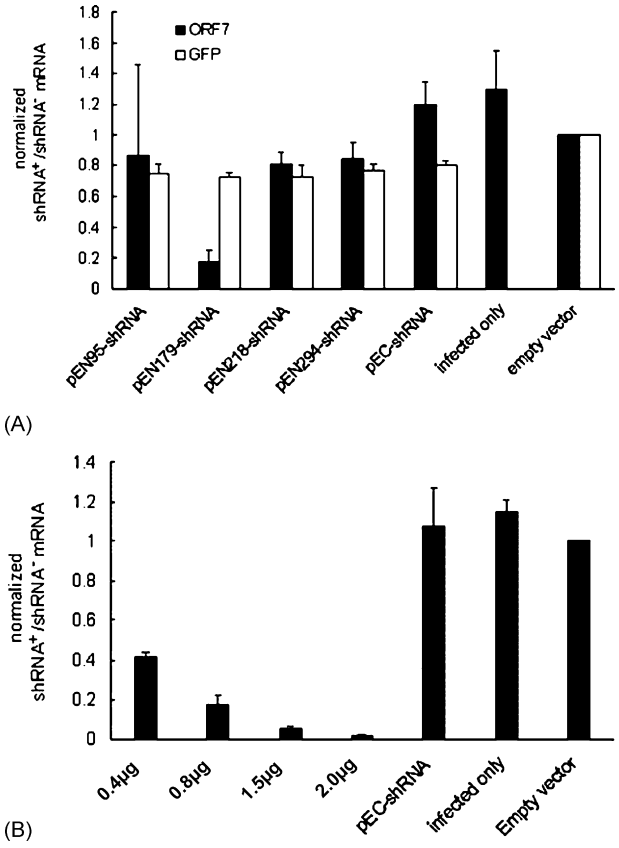

Fig. 5. N-specific small interference RNA (siRNA) inhibits the accumulation of ORF7 message. MARC-145 cells were transfected with variant short hairpin RNA (shRNA)-expressing vectors and then infected with PRRSV at an MOI of 0.01. Sixty hours post-infection, total RNA was extracted and subjected to fluorescence quantitative PCR analysis. pEC-shRNA-transfected cells and mock-transfected cells were used as controls. The mRNA of beta actin served as an internal reference. The mRNA of green fluorescein protein (GFP) was used to monitor transfection level. (A) Black bars indicate normalized $shRNA^+$ (cells transfected with shRNA expression vectors)/$shRNA^-$ (cells transfected with empty vector) ORF7 message copies ratios; white bars indicate normalized $shRNA^+$ (cells transfected with shRNA expression vectors)/$shRNA^-$ (cells transfected with empty vector) GFP message copies ratios. (B) Dose-dependent inhibitory effect of shRNA expression vector. Black bars indicate normalized $shRNA^+$ (cells transfected with different concentration of N179-shRNA expression vectors)/$shRNA^-$ (cells transfected with empty vector) ORF7 message copies ratios. The data shown represent average from three experiments with the standard deviations indicated by error bars.

1.12E + 08, 5.1E + 08, 7.0E + 08, 1.22E + 08, 2.80E + 08, respectively) suggested that the reductions in ORF7 message did not result from poor transfection, nonspecific inhibition or toxicity, because the average mRNA levels of β-actin and EGFP for experimental cells were not significantly reduced compared to the control cells. In addition, the suppressive effect was found to be gene-specific, because the inhibitory effect of empty vector and nonspecific shRNA expression vector pEC-shRNA were negligible. The RNA message of ORF7 in MARC-145 cells transfected with 0.4, 0.8, 1.5 and 2.0 μg of pEN179-shRNA were monitored by FQ-PCR. Fig. 5B shows that at high concentrations (2.0 μg pEN179-shRNA in 1 ml medium) mRNA of ORF7 gene was inhibited by 98%, whilst in the low concentration (0.4 μg pEN179-shRNA in 1 ml medium) mRNA of ORF7 gene was inhibited by 58%, according to Bliss's method, the 50% effective concentration (EC50) for pEN179-shRNA was approximately 0.34 μg/ml.

3.5. Interference of PRRSV replication by shRNA expression vector

The ability of shRNA to inhibit PRRSV replication was determined by titrating the media from the infected cells which had been pre-transfected with shRNA expression vectors. We found that the virus titer of pEN179-shRNA pre-transfected MARC-145 cells was 681-fold lower than that of empty vector controls (Fig. 6). In contrast, transfection with pEN95-shRNA, pEN218-shRNA and pEN294-shRNA reduced the virus titres at 18-, 4- and 11-folds, respectively.

4. Discussion

RNAi is a process of sequence-specific, post-transcriptional gene silencing that is initiated by double stranded RNA. In plants, it is a natural antiviral defense mechanism. In mammalian cells, however, dsRNAs longer than 30 nt activate an antiviral defense leading to the nonspecific degradation of RNA transcripts and a general shutdown of host-cell protein translation (Baglioni and Nilsen, 1983). But 21–23 nt dsRNA is short enough to evade the adverse effects of long dsRNAs and is long enough to mediate gene-specific suppression (Elbashir et al., 2001; Huelsmann et al., 2005). The successful use of siRNA in mammalian cells encouraged the development of siRNA expression vector (Miyagishi and Taira, 2002) and many studies have

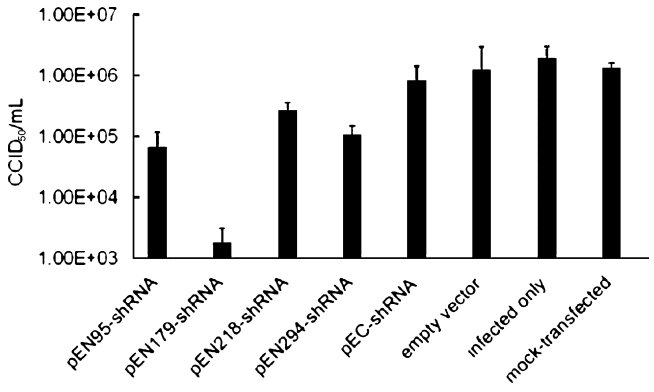

Fig. 6. siRNAs interfere with porcine reproductive and respiratory syndrome virus (PRRSV) production on MARC-145 cells. Supernatants collected from 60 h PRRSV infected cells were serially diluted 10-fold and dispensed into a 96-well plate in triplicate. Five days post-infection, the 50% cell culture infectious dose (CCID50) values were calculated by the Spearman–Karber method. Virus titers are given as $CCID_{50}$/ml (the titers on *y*-axis are shown as scientific notation values). The data shown represents the mean of three experiments, with standard deviations indicated by error bars. The titers on *y*-axis are shown as logarithmic values.

demonstrated that DNA-based siRNA is a promising tool for antiviral therapy in mammalian. Ge et al. (2004) have shown that shRNA transcribed from DNA vectors can reduce virus replication in the lungs of mice, demonstrating a therapeutic effect of siRNAs. Chen et al. (2004) injected the plasmids encoding foot-and-mouth disease virus (FMDV) VP1-specific siRNA into suckling mice and demonstrated this treatment rendered mice less susceptible to FMDV and promoted their survival. Recently, they demonstrate that adenovirus-mediated FMDV-specific shRNA can significantly reduce the susceptibility of guinea pigs and swine to FMDV infection (Chen et al., 2006). Carmona et al. (2005) demonstrated Pol III U6 promoter-encoded shRNAs that target conserved sequences of the oncogenic HBx open reading frame showed knockdown of HBV markers by 80–100% in transfected hepatocytes and also in a murine hydrodynamic injection model of HBV replication. Several groups have developed lentiviral vectors to confer long-term expression of transgenes and modified delivery method to improve RNAi effect, which make RNAi promising in antivirus therapy (Nishitsuji et al., 2004; Rubinson et al., 2003).

Different siRNA sequences display widely differing efficacies, requiring screening of multiple sequences (Holen et al., 2002; Scherer and Rossi, 2004; Scherer and Rossi, 2003). Presently, there are no reliable methods to select effective siRNA sequences without empirically testing, though several groups have proposed a set of guidelines that can narrow the choices of potential siRNAs (Amarzguioui and Prydz, 2004; Boese et al., 2005; Reynolds et al., 2004; Santoyo et al., 2005; Ui-Tei et al., 2004). In this study, we have selected four target sequences for RNA interference using the software applications, "siRNA Target Finder and Design Tool" available at http://www.ambion.com/. As the ORF7 gene is well conserved in a given type of PRRSV and N protein is the most abundance protein in PRRSV virion, we selected ORF7 as a target gene. In order to generate shRNA expression cassettes quickly and accurately, we employed a PCR-based strategy to clone siRNA sequences. In this strategy, siRNA sequences were designed as a single primer sequence onto which 19 nt of sequence complement to the mouse U6 promoter were added. The resulting PCR products are shRNA expression cassettes including mouse U6 promoter. shRNAs that have been generated from this expression system are efficiently processed by dicer into siRNAs. To simplify the cloning process, the *Eco*O109I restriction site sequences were added to forward and reverse primers (Fig. 1B). With this design, we can insert the cassettes to different vectors. In this study, we selected pEGFP-N1 vector which contains an EGFP gene as report gene and an SV40 origin for replication in mammalian cells. It has been demonstrated that the 9 nt loop in hairpin RNA is the most effective and the corresponding shRNA-expressing vector that is able to knockdown gene expression to the same extent as that of synthetic siRNA (Brummelkamp et al., 2002b; Castanotto et al., 2002).

Of these four targets, N179 was found to be highly effective, while N95, N294 and N218 displayed weak activity. A potential explanation for the failure of these sequences is that the first nucleotide of N95 and N218 is not guanine (G) while the U6 promoter is known to initiate transcription starting from the first G (Elbashir et al., 2002). Furthermore, according to the guidelines reported by Ui-Tei et al. (2004), the 7 bp antisence terminal duplex regions of highly effective and ineffective siRNAs are AU rich and GC rich, respectively. With effective siRNAs, the A/U at the 5′ antisense end of the sequence may possibly be required not only for target recognition but also for RISC formation as well, which includes siRNA unwinding. The putative siRNA helicase preferably initiates unwinding of the RNA duplex in an AU-rich terminal region with A/U at its 5′-free end, while RNA duplex unwinding from the GC-rich terminal region with G/C at its 5′-free end is blocked. siRNAs opposite in features with this conditions bring about little or no gene silencing. When comparing our four siRNA sequences to these guidelines, only N179 stringently meets these rules and it is very interesting. In addition, as we did not use a scrambled sequence of active shRNA to guarantee the specific effect, we did not rule that the inhibition was due to nonspecific effects associated with the particular bases contained in the active shRNA.

MARC-145 cells transfected with pEN95-shRNA, pEN179-shRNA, pEN218-shRNA, pEN294-shRNA, pEC-shRNA and controls were examined for CPE, indirect immunofluorescence assay, FQ-PCR and virus titration. All results demonstrated that N179 site is the most effective RNAi target site.

Blasting N179 sequence in GenBank revealed that there were 16 PRRSV isolates containing identical sequence corresponding to N179. Sequence analysis revealed that all of the 16 isolates were North American type (date not shown). In view of the sequences of the N genes of PRRSV strains from the same genotype, they all share high homology (96–99%). Therefore, N gene is a good target to suppress PRRSV replication by RNAi.

PRRSV is a positive-stranded RNA virus. It has been reported that plus-strand RNA viruses appear to be attractive targets for siRNAs, as their genome functions as both mRNA and replication template (Ge et al., 2003; Kanda et al., 2004). Perhaps a similar phenomenon is occurring here and the RNA genome of PRRSV is itself a target of this siRNA.

In conclusion, this study demonstrates that vector-based shRNA methodology can effectively inhibit PRRSV replication on MARC-145 cells. Further study will be required to determine whether such treatment could protect against PRRSV infection in vivo. Still, this work represents a significant advance, potentially facilitating new experimental approaches in the analysis of both viral and cellular gene functions in the context of PRRSV infection.

Acknowledgments

The study was supported by grants from the National Natural Science Foundation of China (no. 30470072) and the National Basic Research Program (973) of China (no. 2005CB523200).

References

Amarzguioui, M., Prydz, H., 2004. An algorithm for selection of functional siRNA sequences. Biochem. Biophys. Res. Commun. 316, 1050–1058.

Baglioni, C., Nilsen, T.W., 1983. Mechanisms of antiviral action of interferon. Interferon 5, 23–42.

Bautista, E.M., Meulenberg, J.J., Choi, C.S., Molitor, T.W., 1996. Structural polypeptides of the American VR-2332 strain of porcine reproductive and respiratory syndrome virus. Arch. Virol. 141, 1357–1365.

Boese, Q., Leake, D., Reynolds, A., Read, S., Scaringe, S.A., Marshall, W.S., Khvorova, A., 2005. Mechanistic insights aid computational short interfering RNA design. Methods Enzymol. 392, 73–96.

Brummelkamp, T.R., Bernards, R., Agami, R., 2002a. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell 2, 243–247.

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

Carmona, S., Ely, A., Crowther, C., Moolla, N., Salazar, F.H., Marion, P.L., Ferry, N., Weinberg, M.S., Arbuthnot, P., 2005. Effective inhibition of HBV replication in vivo by anti-HBx short hairpin RNAs. Mol. Ther..

Castanotto, D., Li, H., Rossi, J.J., 2002. Functional siRNA expression from transfected PCR products. RNA 8, 1454–1460.

Cavanagh, D., 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch. Virol. 142, 629–633.

Chen, W., Liu, M., Jiao, Y., Yan, W., Wei, X., Chen, J., Fei, L., Liu, Y., Zuo, X., Yang, F., Lu, Y., Zheng, Z., 2006. Adenovirus-mediated RNA interference against foot-and-mouth disease virus infection both in vitro and in vivo. J. Virol. 80, 3559–3566.

Chen, W., Yan, W., Du, Q., Fei, L., Liu, M., Ni, Z., Sheng, Z., Zheng, Z., 2004. RNA interference targeting VP1 inhibits foot-and-mouth disease virus replication in BHK-21 cells and suckling mice. J. Virol. 78, 6900–6907.

Collins, J.E., Benfield, D.A., Christianson, W.T., Harris, L., Hennings, J.C., Shaw, D.P., Goyal, S.M., McCullough, S., Morrison, R.B., Joo, H.S., et al., 1992. Isolation of swine infertility and respiratory syndrome virus isolate ATCC VR-2332 in North America and experimental reproduction of the disease in gnotobiotic pigs. J. Vet. Diagn. Invest. 4, 117–126.

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.

Ge, Q., Filip, L., Bai, A., Nguyen, T., Eisen, H.N., Chen, J., 2004. Inhibition of influenza virus production in virus-infected mice by RNA interference. Proc. Natl. Acad. Sci. USA 101, 8676–8681.

Ge, Q., McManus, M.T., Nguyen, T., Shen, C.H., Sharp, P.A., Eisen, H.N., Chen, J., 2003. RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc. Natl. Acad. Sci. USA 100, 2718–2723.

Guo, B.Q., Chen, Z.S., Liu, W.X., Cui, Y.Z., Kong, L.D., 1996. Isolation of a porcine reproductive and respiratory syndrome (PRRS) virus from aborted fetus suspected of PRRS. Chin. J. Infect. Dis. Anim. Poult. 18, 1–5.

Hammond, S.M., 2005. Dicing and slicing: the core machinery of the RNA interference pathway. FEBS Lett. 579, 5822–5829.

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.

Huelsmann, P.M., Rauch, P., Allers, K., John, M.J., Metzner, K.J., 2005. Inhibition of drug-resistant HIV-1 by RNA interference. Antiviral Res. 69, 1–8.

Kaerber, G., 1931. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Naunyn Schmiedbergs Arch. Exp. Pathol. Pharmakol. 162, 480–483.

Kanda, T., Kusov, Y., Yokosuka, O., Gauss-Muller, V., 2004. Interference of hepatitis A virus replication by small interfering RNAs. Biochem. Biophys. Res. Commun. 318, 341–345.

Mardassi, H., Mounir, S., Dea, S., 1994. Identification of major differences in the nucleocapsid protein genes of a Quebec strain and European strains of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 75 (Pt 3), 681–685.

McCaffrey, A.P., Nakai, H., Pandey, K., Huang, Z., Salazar, F.H., Xu, H., Wieland, S.F., Marion, P.L., Kay, M.A., 2003. Inhibition of hepatitis B virus in mice by RNA interference. Nat. Biotechnol. 21, 639–644.

Meulenberg, J.J., de Meijer, E.J., Moormann, R.J., 1993a. Subgenomic RNAs of Lelystad virus contain a conserved leader-body junction sequence. J. Gen. Virol. 74 (Pt 8), 1697–1701.

Meulenberg, J.J., Hulst, M.M., de Meijer, E.J., Moonen, P.L., den Besten, A., de Kluyver, E.P., Wensvoort, G., Moormann, R.J., 1993b. Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome PEARS, is related to LDV and EAV. Virology 192, 62–72.

Miyagishi, M., Taira, K., 2002. U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. Nat. Biotechnol. 20, 497–500.

Nelson, E.A., Christopher-Hennings, J., Drew, T., Wensvoort, G., Collins, J.E., Benfield, D.A., 1993. Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. J. Clin. Microbiol. 31, 3184–3189.

Nishitsuji, H., Ikeda, T., Miyoshi, H., Ohashi, T., Kannagi, M., Masuda, T., 2004. Expression of small hairpin RNA by lentivirus-based vector confers efficient and stable gene-suppression of HIV-1 on human cells including primary non-dividing cells. Microbes Infect. 6, 76–85.

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.

Pejsak, Z., Markowska-Daniel, I., 1997. Losses due to porcine reproductive and respiratory syndrome in a large swine farm. Comp. Immunol. Microbiol. Infect. Dis. 20, 345–352.

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.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W.S., Khvorova, A., 2004. Rational siRNA design for RNA interference. Nat. Biotechnol. 22, 326–330.

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.

Santoyo, J., Vaquerizas, J.M., Dopazo, J., 2005. Highly specific and accurate selection of siRNAs for high-throughput functional assays. Bioinformatics 21, 1376–1382.

Scherer, L., Rossi, J.J., 2004. Therapeutic applications of RNA interference: recent advances in siRNA design. Adv. Genet. 52, 1–21.

Scherer, L.J., Rossi, J.J., 2003. Approaches for the sequence-specific knockdown of mRNA. Nat. Biotechnol. 21, 1457–1465.

Sharp, P.A., Zamore, P.D., 2000. Molecular biology. RNA interference. Science 287, 2431–2433.

Snijder, E.J., van Tol, H., Pedersen, K.W., Raamsman, M.J., de Vries, A.A., 1999. Identification of a novel structural protein of arteriviruses. J. Virol. 73, 6335–6345.

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

Sui, G., Soohoo, C., Affar el, B., Gay, F., Shi, Y., 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.

Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., Ueda, R., Saigo, K., 2004. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res. 32, 936–948.

Verheije, M.H., Kroese, M.V., Rottier, P.J., Meulenberg, J.J., 2001. Viable porcine arteriviruses with deletions proximal to the 3′ end of the genome. J. Gen. Virol. 82, 2607–2614.

Wensvoort, G., 1993. Lelystad virus and the porcine epidemic abortion and respiratory syndrome. Vet. Res. 24, 117–124.

Wu, W.H., Fang, Y., Farwell, R., Steffen-Bien, M., Rowland, R.R., Christopher-Hennings, J., Nelson, E.A., 2001. A 10-kDa structural protein of porcine reproductive and respiratory syndrome virus encoded by ORF2b. Virology 287, 183–191.

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

Zhou, Y.J., Xue, Q., An, T.Q., Qiu, H.J., Tong, G.Z., Wang, Y.F., Tian, Z.J., Wang, M., Cai, X.H., 2005. Development of monoclonal antibodies against nucleocapsid protein of PRRSV CH-1a strain. Chin. J. Prevent. Vet. Med. 27 (6), 498–503.