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In vitro inhibition of CSFV replication by retroviral vector-mediated RNA interference

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ABSTRACT

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Classical swine fever (CSF) is a highly contagious viral disease of pigs which causes major economic losses worldwide. No specific drug is currently available for the effective treatment of CSFV infection. RNA interference (RNAi) technology depends on effective delivery systems, for which several effective vectors have recently been developed. Three retroviral plasmids containing siRNA genes targeting different regions of *N^{pro}* and *NS4A* have been constructed, and 3 replication-incompetent retroviral vectors have been produced in the human embryo kidney cell line GP2-293 by retroviral plasmid transfection. PK-15 cells were then infected with these replication-incompetent retroviral vectors and screened for siRNA stably expressing PK-15 cell clones. Growth of CSFV in such siRNA stably expressing cell clones resulted in a 186-fold reduction in viral genome copies and, at 72 h post-infection, only a small % of cells showed infection by indirect immunofluorescence microscopy, and effective inhibition of virus replication persisted for up to 120 h. Retroviral vector-mediated RNAi can therefore be used to study the specific function of viral genes associated with CSFV replication and may have potential therapeutic application.

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1. Introduction

RNA interference (RNAi) is a natural posttranscriptional gene silencing mechanism, which is induced by 19–27 nucleotide (nt) small interfering RNA (siRNA) molecules homologous to the target genes (Jana et al., 2004). Since its discovery, RNAi has been regarded by virologists as a promising method for suppression of viral infection, and has been applied successfully to inhibit the replication of some human and animal viruses, including HIV-1, hepatitis C virus, hepatitis B virus and SARS-CoV, both *in vitro* and *in vivo* (Ma et al., 2007).

Classical swine fever (CSF) is a highly contagious disease of pigs caused by infection with CSFV, and is a notifiable diseases of the World Organization for Animal Health (OIE). CSF causes great economic losses in the pig industry worldwide, particularly in Asia, Latin America, and Eastern Europe. The disease was first recognized in China in the 1920s and there continue to be major epizootics in China (Tu et al., 2001; Zhu et al., 2009). CSFV belongs to the genus *Pestivirus* of the family *Flaviviridae*. Its genome consists of a single-stranded (+) sense RNA of about 12.5 kb with a single large open reading frame (ORF), encoding a polyprotein (Meyers et al., 1989). After translation, the polyprotein is cleaved into viral structural and

non-structural peptides which are, from N- to C-terminus, *N^{pro}*-C-E^{ms}-E1-E2-P7-NS2-NS3-NS4A-NS4B-NS5A-NS5B (Meyers et al., 1989; Elbers et al., 1996; Ruggli et al., 1996; Tautz et al., 1997). *N^{pro}* is an atypical cysteine protease with an autoproteolytic activity that cleaves the viral polyprotein via cis hydrolysis, generating the N-terminus of the capsid protein C (Rumenapf et al., 1998). *N^{pro}* is also essential for evading the cellular antiviral defense system, and protects cells from double-stranded RNA-induced apoptosis (Ruggli et al., 2005). *NS4A* is an indispensable cofactor of uncleaved *NS2-3* in the formation of infectious particles, with the N-terminus of *NS3* providing serine protease activity (Moulin et al., 2007).

Previous study of *in vitro*-transcribed siRNA molecules targeting *N^{pro}* and *NS5B* for their repressive effects on CSFV replication (Xu et al., 2008) showed that 21-nt siRNAs are capable of specific and efficient inhibition of CSFV replication. While RNAi has potential as an antiviral strategy against CSFV in animal systems, *in vitro* transcribed siRNA has some limitations for this application. To overcome these, siRNA delivery systems using retroviral (Ma et al., 2007; Schuck et al., 2004), adenoviral (Chen et al., 2006) and lentiviral vectors (Miest et al., 2009) have been reported. Retroviral delivery is well-recognized as an efficient means of introducing genetic material into cells, and effective in most cell cultures, and has been used in generating transgenic animals (Barquinero et al., 2004). By combining a vector-based siRNA-generating system with retroviral delivery, highly efficient transfection and stable suppression of specific gene expression are possible. To explore a

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Table 1
List of shRNA sequences in this study.

Target name ^a	Sequences of shRNA (5' to 3') ^b
N1 S	GACGGATCCGGATAGGTAGGGTGACAGGTTCAAGAGACCTGTACACCTACCTATCCTTTTTGGAAAAGCTTGCA
N1 AS	TGCAAGCTTTTCCAAAAAAGGATAGGTAGGGTGACAGGTTCTTGAACCTGTACACCTACCTATCCGGATCCGTC
N2 S	GACGGATCCGAACCTGAAGTGGATTAGTTCAGAGACTAATCCACTTCAGGGTTCTTTTTGGAAAAGCTTGCA
N2AS	TGCAAGCTTTTCCAAAAAAGAACCTGAAGTGGATTAGTCTTGAACCTAATCCACTTCAGGGTTCCGGATCCGTC
S11 S	GGAGGATCCGAGGCATATACCAGTAGTCATTCAAGAGATGACTACTGGTATATGCCTTTTTGGAAAAGCTTCGG
S11 AS	CCGAAAGCTTTTCCAAAAAAGAGGCATATACCAGTAGTCATCTTGAATGACTACTGGTATATGCCTCGGATCCCTCC
C S	GGAGGATCCGACAGTGGGATGGATAGGTTCAAGAGACCTATCCATCCCACTGTCTTTTTGGAAAAGCTTCGG
C AS	CCGAAAGCTTTTCCAAAAAAGACAGTGGGATGGATAGGTTCTTGAACCTATCCATCCCACTGTCCGGATCCCTCC

^a S means sense strand, AS means antisense strand, C means control shRNA sequence.

^b For each target gene (sense strand as an example), complementary 75-mer oligonucleotides were designed with 5' single-stranded overhangs (BamH I) and 3' single-stranded overhangs (Hind III) for ligation into the pSilencer vectors. Sequences for enzyme sites are underlined. The oligonucleotides should encode 19-mer hairpin sequences specific to the mRNA target, a loop sequence separating the two complementary domains (TTCAAGAGA), and a polythymidine tract to terminate transcription (TTTTTT).

new approach for prevention and therapy of classical swine fever, retroviral vector-mediated RNA interference was studied for its inhibitory effects on CSFV replication in PK-15 cells. Results showed that siRNAs were stably integrated into target cells and inhibited efficiently CSFV replication *in vitro*. This study therefore provides not only an experimental basis for the development of a promising anti-CSFV method, but also for a new approach to the study of viral infection and replication.

2. Materials and methods

2.1. Cells, virus, and sera

Pig kidney cell line PK-15 (80 passages) and the virulent CSFV strain Shimen were obtained from the Institute of Veterinary Drug Control, China. Positive anti-CSFV serum and negative control serum were prepared as described previously (Yu et al., 2001).

2.2. Plasmids construction

Sequences from the *N^{pro}* and *NS4A* genes (GenBank accession: AF092448) were scanned for the signature sequence AA-N₁₉. Two designed 21 nt candidate sequences, siN1 and siN2, as described previously (Xu et al., 2008), were chosen as siRNAs for *N^{pro}*. The RNAi target within *NS4A* was also selected in the study, with the sequence depicted below. Sequences selected for a negative siRNA control (siC) were the same as in a previous study (Xu et al., 2008). For each sequence, oligo DNA was synthesized by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. (Shanghai, China). The sequences are shown in Table 1.

These oligonucleotides were annealed and ligated to the BamH I and Hind III sites of pSilencer3.1H1 (Ambion, Austin, TX, USA) to

get plasmids pH1-(siRNAs), where “siRNAs” represents siN1, siN2, siS11 and siC. To get H1 promoter, H1-(siRNAs) were amplified by PCR using plasmids pH1-(siRNAs) as templates, with the following primers: pH1-fw and pH1-rev (shown in Table 2).

H1-(siRNAs) were digested and ligated to the Xho I sites of pLNHX (Clontech, Mountain View, CA, USA) to form plasmids pLN-(siRNAs). The insert sequences were confirmed by sequencing by Shanghai Sangon Biological Engineering Technology & Service Co.

2.3. Production of recombinant retrovirus

The packaging cell line GP2-293 (Clontech, Mountain View, CA, USA) was grown in DMEM with 10% FCS (GIBCO)/10 mM HEPES/2 mM L-glutamine/1 mM MEM sodium pyruvate/100 U/mL penicillin/100 µg/mL streptomycin. The cells in 100-mm dishes were transfected by FuGENE HD (Roche, Indianapolis, IN, USA) with 6 µg pVSV-G (Clontech, Mountain View, CA, USA) and 10 µg pLN-(siRNAs). Forty-eight hours post-transfection, each supernatant was collected, filtered through a 0.45-µm syringe filter, and assayed.

2.4. Screening for siRNA-expressing cells and CSFV infection

Wells of 100-mm cell culture dishes were seeded with 3 × 10⁶ PK-15 cells. The next day, the cells were incubated with 10⁴ cfu (colony forming units) per well recombinant retrovirus containing polybrene (8 µg/mL final concentration) for 2 h at 37 °C, following which the medium was replaced. After 12 h, the medium was removed and replaced with fresh medium containing G418 (500 µg/mL, GIBCO). This was repeated 3 times at 3-day intervals. On day 14, colonies of G418-resistant monoclonal cells were screened for stable expression of siRNAs. The cell clones were cul-

Table 2
Primers and probe used in this study.

Primer name	Sequences (5' to 3')
pH1-fw	CITCTCGAGGGTTTTCCAGTCACGAC
pH1-rev	GCGCTCGAGGCACCCAGGCTTTACACTTT
pLN-fw	GAAAGGTTGGGCTTCGGAATC
pLN-rev	CTTTAAACAAATTGGACTAATC
FP147	GCTCCCTGGTGGTCTAAGTC
RP267	CGCYAGGGTTAAGGTGTGTCTTG
Probe187	FAM-CAGTAGTTCGACGTGAGCAGAAGCCAC-TAMRA
RT6-siN1	TGTCAGGCAACCGTATTACCGTGAGTGGTAAGGAT
RT6-siN2	TGTCAGGCAACCGTATTACCGTGAGTGGTAAGAAC
RT6-siS11	TGTCAGGCAACCGTATTACCGTGAGTGGTAATGAC
Short-siN1-rev	CGTCAGATGTCCGAGTAGAGGGGGAACGGCGCTGTACCCTACCT
Short-siN2-rev	CGTCAGATGTCCGAGTAGAGGGGGAACGGCGCTAATCCACTCAGG
Short-siS11-rev	CGTCAGATGTCCGAGTAGAGGGGGAACGGCGGAGGCATATACCAG
MP-fw	TGTCAGGCAACCGTATTACCC
MP-rev	CGTCAGATGTCCGAGTAGAGG
5S rRNA-fw	GCCCGATCTCGTCTGATCT
5S rRNA-rev	AGCCTACAGCACCCGGTATT

tured, infected with 500 TCID₅₀ of CSFV (strain Shimen) per well in 24-well plates, and incubated at 37 °C for the various time periods indicated. Infected cells were identified by indirect immunofluorescence assay, and infectious virus production was determined by TCID₅₀ assay, as previously described (Xu et al., 2008). The copy number of CSFV genomic RNA was determined by real-time RT-PCR. Integrated siRNAs, H1-(siRNAs), were amplified by nested-PCR, and the siRNA expression analysis were detected by RT-PCR.

2.5. Real-time RT-PCR quantitation of viral genome

2.5.1. Primer and probe

To use the 5'-UTR of the viral genome as a quantitative RT-PCR standard, primers were designed for CSFV strain Shimen using Vector NTI 3.0 (Amersham, Piscataway, NJ, USA). Three primers were synthesized for this purpose: FP147, RP267 and probe187 (shown in Table 2). The target region for real-time RT-PCR was nt 147–267 of 5'-UTR.

2.5.2. Construction of 5'-UTR plasmid

Total RNA of CSFV-infected cells was extracted with Trizol LS Reagent (Invitrogen, Carlsbad, CA, USA) and then applied as a template for synthesis of the first cDNA strand of the 5'-UTR by RT-PCR using AMV reverse transcriptase (Promega, Madison, WI, USA) and 5'-UTR RP267 primer, according to the manufacturer's instructions. PCR was then used to amplify the 5'-UTR with FP147 and RP267 primers under conditions of 94 °C for 2 min, then 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 20 s, with a final extension at 72 °C for 7 min. The PCR product was gel-purified using the Agarose Gel DNA Extraction Kit (Axygen, Union, CA, USA) and then cloned into pGEM-T vector (Promega, Madison, WI, USA). The resulting plasmid, pT-5'-UTR, with the correct sequence confirmed by direct sequencing, was selected as a quantitative standard for determination of the viral RNA copy number. The plasmid was prepared and stored at -80 °C after determination of its concentration by the GeneQuant RNA/DNA Calculator.

2.5.3. Real-time PCR

For quantitative analysis of the CSFV genome, 100 ng total RNA from CSFV-infected cells was mixed with 1 μL RP267 primer, heated to 65 °C for 5 min and chilled on ice for 2 min. To this primer-template mix was added 5× buffer (4 μL), 10 mmol/L dNTP (1 μL), RNasin (1 μL), AMV reverse transcriptase (1 μL, Promega, Madison, WI, USA) and ddH₂O to a total volume of 20 μL. The reaction mixture was incubated at 42 °C for 45 min, followed by inactivation of reverse transcriptase at 75 °C for 15 min. Real-time PCR was performed with the ABI PRISM® 7000 Sequence Detection System using 2 μL transcriptase products as template under the conditions of 95 °C for 15 min, followed by 50 cycles of denaturation at 95 °C for 30 s, annealing, and extension at 60 °C for 30 s. The quantitative standard curve for determination of CSFV genome copy number was created by real-time PCR of standard plasmid pT-5'-UTR serial 10-fold dilutions of a stock containing 10⁸ copies/μL. The specificity of the real-time PCR was confirmed by sequencing of the product.

2.6. Analysis of siRNA gene integration

Genomes were extracted from siRNA stably expressing cells at passages 5 and 15. Integrated siRNAs, H1-(siRNAs), were amplified by nested-PCR, with pLN-fw and pLN-rev as outer set primers, and pH1-fw and pH1-rev as inner set primers (shown in Table 2).

PCR was used to amplify the H1-(siRNAs) genes under conditions of 94 °C for 10 min, then 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 20 s, with a final extension at 72 °C for 7 min. The PCR products were detected by 1.2% agarose gel electrophoresis, and

the specificity of the PCR was confirmed by sequencing of the inner set product.

2.7. Analysis of siRNA expression by RT-PCR

RNA from siRNA stably expressing cell clones were prepared using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA), according to the manufacturer's protocol. One hundred ng aliquots of sample RNA were mixed with reverse transcription primer RT6-X, where "X" represents siN1, siN2 and siS11 (shown in Table 2). To this primer-template mix was then added 5× buffer (4 μL), 10 mmol/L dNTP (1 μL), RNasin (1 μL), AMV reverse transcriptase (1 μL, Promega, Madison, WI, USA) and water to a total volume of 20 μL. RT reaction was kept at 16 °C for 30 min then at 42 °C for 45 min before it was terminated by incubating the mixture at 75 °C for 5 min.

PCR was then used to amplify with the primers Short-X-rev, MP-fw and MP-rev, under conditions of 94 °C for 2 min, then 35 cycles of 94 °C for 15 s, 58 °C for 5 s and 72 °C for 15 s. Synthetic RNA-oligonucleotides siN1 were used for the positive control of assays. The positive control assay was performed by RT of 50 ng of the synthetic siN1 mixture with 50 ng RNA from PK-15 cells present, and RNA from PK-15 cells as negative control. 5S rRNA were chosen as a housekeeping gene with primers 5S rRNA-fw and 5S rRNA-rev (shown in Table 2). The method has been described previously (Sharbati-Tehrani et al., 2008). The PCR products were detected by 15% polyacrylamide gel electrophoresis.

2.8. Statistical analysis

All numerical parameters, including genome copies and viral titers, are expressed as means ± standard deviations. Student's *t* test was used for the analysis of numeric parameters (SPSS 13.0 for Windows, SPCC Inc.). All differences were considered significant at *P* values of <0.01.

3. Results

3.1. Examination of siRNA effect by IFA

To study the inhibitory effects of RNA interference on CSFV replication, the level of viral antigen produced in siRNA stably expressing cells was examined by IFA using anti-CSFV serum after viral infection. At 72 h post-CSFV infection, most siC stably expressing cells exhibited bright green fluorescence in the cytoplasm, indicating that most cells in the control were producing virus (Fig. 1). By contrast, only few cell stably expressing siN1, siN2 or siS11 displayed green fluorescence, indicating that most were effectively protected by the siRNA and resisted viral infection. With increasing time the level of viral antigen increased, but to a lower level than the control.

3.2. Examination of siRNA effect by real-time RT-PCR

To quantitate the effect of siRNA on viral replication, the viral genome copy number was determined by real-time PCR, using serially diluted plasmid pT-5'-UTR as a standard. The *R*² value of the standard curve was 0.99 and the average amplification efficiency *E* was 0.990, calculated from the formula $E = 10^{-1/\text{slope}} - 1$. These data indicate that the real-time PCR was highly reliable in the assay, and sequencing showed that the fragment amplified was the expected part of 5'-UTR gene, thereby demonstrating the specificity and reliability of the analysis. As shown in Fig. 2, at 48 h and 60 h post-viral infection, the copy number of the viral genome per ng total RNA calculated from the standard curve was 3.93×10^4 , 5.33×10^4 copies/ng of the viral genome in the total RNA from siC

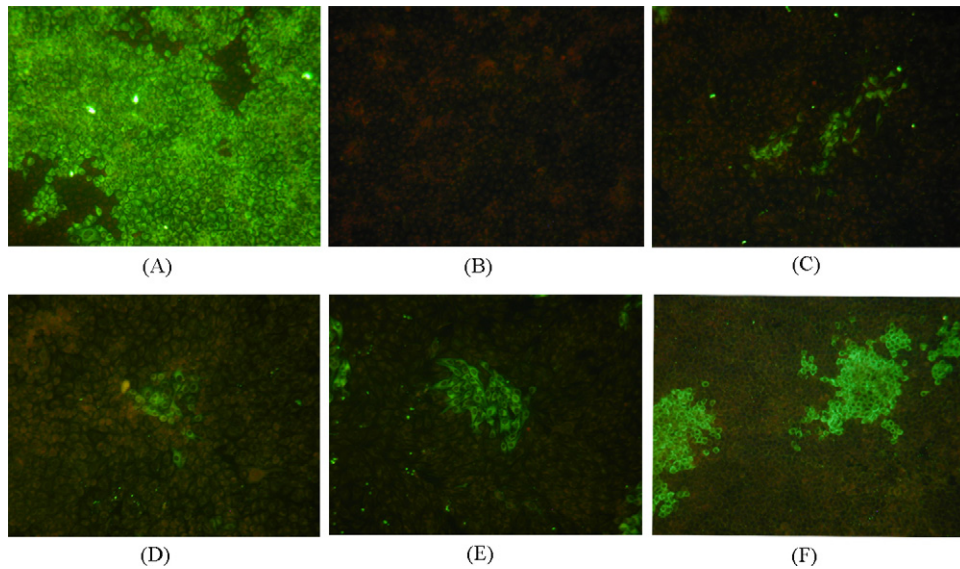


Fig. 1. Inhibition of CSFV replication by the siRNAs at 72 h post-CSFV infection. (A) siC (siC stably expressing cell clone with CSFV infection). (B) Negative control (siC stably expressing cell clone without CSFV infection). (C) siN1 (siN1 stably expressing cell clone with CSFV infection). (D) siN2 (siN2-stably expressing cell clone with CSFV infection). (E) siS11 (siS11 stably expressing cell clone with CSFV infection). (F) siN2 (120 h post-infection).

stably expressing cells, followed by a gradual decrease in titer from 72 to 120 h post-infection. At 48 h post-infection, the copy numbers were 2.10×10^2 , 2.62×10^2 , 1.93×10^3 /ng in siN1, siN2 and siS11 stably expressing cells, respectively, corresponding to 186, 149 and 19-fold reductions. With increasing time the copy numbers of the viral genome increased, but at 120 h post-infection were still 2–15-fold lower than in siC stably expressing cells.

3.3. Examination of siRNA effect by infectious virus assay

The TCID₅₀ assay was performed to examine the effect of siRNA on production of viable virus. Fig. 3 shows that titers of CSFV reached $10^{4.33}$, $10^{4.50}$, $10^{4.58}$ TCID₅₀/mL at 48 h, 60 h and 72 h post-infection, respectively in siC stably expressing cells, before gradual decreasing. In contrast, titers at 48 h post-infection were $10^{2.58}$, $10^{2.17}$ and $10^{2.97}$ TCID₅₀/mL in siN1-, siN2- and siC-stable expression cells, corresponding to 55-, 143- and 23-fold reductions, respectively. Titers at 60 h post-infection were $10^{3.42}$, $10^{3.42}$ and $10^{3.33}$ TCID₅₀/mL in siN1-, siN2- and siC-stable expression cells, respectively, corresponding to 12.02, 12.02 and 17.49-fold reductions. The data indicate that the siRNAs markedly inhibited infectious virus production, but with increasing time the level of inhibition decreased.

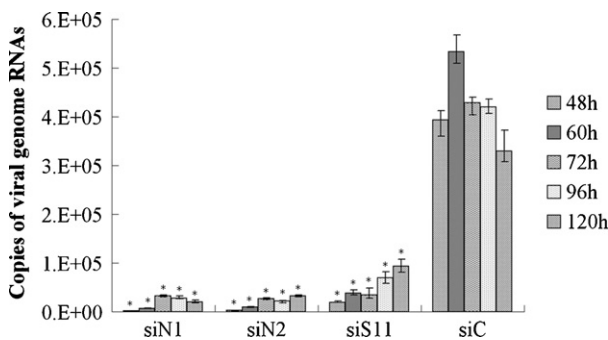


Fig. 2. Genomic replication kinetics of CSFV in siRNA stably expressing PK-15 cells. The CSFV genome copy numbers are the means of three repeat experiments. **P* < 0.01 vs. siC stably expressing PK-15 cells.

3.4. Examination of siRNA gene integration analysis

Amplification of template genomes DNA of siRNA stably expressed cells with the nested-PCR primers resulted in amplified products corresponding to those of the predicted size: 450 (outer set) and 310 (inner set) bp. The PCR inner set products were sequenced, and their identities confirmed as H1-(siRNAs). Products amplified from passages 5 and 15 were identical. These results confirm that siRNAs were stably integrated into the target cell genomes.

3.5. Examination of siRNA expression analysis

RT-PCR was used to test for expression of siRNA. Two siRNAs (siN1 and siN2) and a positive control were amplified, and the products of each were the predicted size (82 bp; Fig. 4) but siS11 failed to produce an amplification product (data not shown). Expression of the 5S rRNA gene was determined as a housekeeping gene, with a product size of 93 bp (Fig. 4). The results indicate that siRNAs were expressed in siN1- and siN2-stably expressing cells.

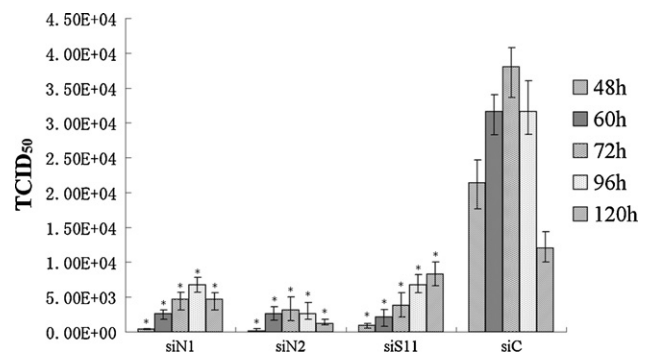


Fig. 3. Viable viral production in three siRNA stably expressing PK-15 cell clones. TCID₅₀ are the means of three repeat titrations at the time points indicated. **P* < 0.01 vs. siC stably expressing PK-15 cells.

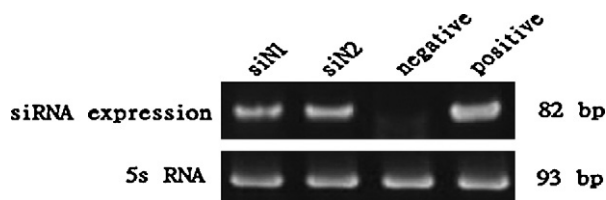


Fig. 4. Detection of siRNAs expression in siRNA stably expressing PK-15 cell clones by RT-PCR.

4. Discussion

siRNAs can be introduced into mammalian cells by various means. First, cells can be transfected with chemically synthesized siRNAs or *in vitro*-transcribed siRNAs (Elbashir et al., 2001; Xu et al., 2008). This can achieve effective and very rapid silencing of a target gene, although limited to cells that can be transfected at high rates and the effects are transient. To circumvent the latter problem, so-called short hairpin RNAs (shRNAs) can be expressed from stably transfected plasmids (Kapadia et al., 2003). Like miRNA precursors, shRNAs form hairpin structures, which are cleaved by Dicer to produce siRNAs. However, the generation of shRNA-expressing cell lines are time-consuming. An alternative way to achieve stable integration of shRNA expression cassettes into the genome of cells is delivery via retroviruses (Schuck et al., 2004). Their production is fast and simple. If expression of shRNAs is combined with expression of a selectable marker, populations in which every cell contains at least one active copy of the retroviral genome can readily be obtained. This study has demonstrated the use of retroviral vector-based RNAi against CSFV, a major pathogen of pigs. Three different siRNA gene constructs, delivered via a replication-competent retroviral vector, successfully reduced viral replication using targets in viral genes *N^{pro}* and *NS4A*, two key genes involved in CSFV replication. The results showed that these siRNAs were stably integrated into, and expressed in PK-15 cells and were capable of significantly inhibiting viral genomic RNA replication.

The CSFV genome contains a single large ORF encoding a polyprotein (Meyers et al., 1989). Since *N^{pro}* is the ultimate N-terminal protease involved in the polyprotein processing, the binding of siRNA to *N^{pro}* mRNA likely interfered with the translation and the subsequent processing of the viral polyprotein, thereby resulting in significant inhibition of the viral replication. Moreover, there are studies showing that it is essential that the sequence downstream of the internal ribosome entry site within the starting region of CSFV mRNA should be single-stranded for high efficiency translation (Ruggli et al., 1996; Wilson et al., 2003). Consequently, since this part of the *N^{pro}* mRNA may not form a secondary structure, it is likely to be highly accessible to siRNA (Kretschmer-Kazemi Far and Sczakiel, 2003). *NS4A* is an indispensable cofactor of uncleaved *NS2-3* in the formation of infectious particles, with the N-terminus of *NS3* having serine protease activity (Moulin et al., 2007). That uncleaved *NS2-3* recruited for virion assembly does not require the enzymatic activity of *NS3* essential for RNA replication further supports the hypothesis that *NS2-3* and *NS3*, each in association with *NS4A*, have independent functions in the CSFV life cycle. Based on this knowledge, satisfactory RNA interference results were obtained by siRNAs generated against both key genes, although no research studying the effect of RNAi on the *NS4A* gene has been done before.

This study has demonstrated the use of retroviral vector-mediated RNAi against CSFV in PK-15 cells. Standard molecular cloning techniques were employed for the construction and generation of 3 different siRNA retroviruses, corresponding to different regions of the CSFV genome: *N^{pro}* and *NS4A*. The retroviruses infected PK-15 cells, and expression of siRNAs was combined with

expression of a selectable marker. siRNA stably expressing cell clones were obtained in which every cell clone contained at least one active copy of siRNA. Knowledge of the kinetics of CSFV replication in cultured cells is critical for determination of the sampling points for estimation of siRNA effect in cells. Hence, the replication kinetics of CSFV strain Shimen in PK-15 cells was studied by IFA and TCID₅₀ assay prior to siRNA transfection, with results showing that the proliferation of virus peaked at 48–72 h post-infection (Xu et al., 2008). Based on this consideration, viral growth in siRNA stable expression cells was determined after 48 h post-infection.

Silencing of CSFV gene expression induced in siRNA stably expressing cells is time-dependent. As shown in Fig. 3, significant elevations in virus titers seen at 120 h post-infection of siRNA stably expressing cells indicates that the effects of these siRNAs diminished after 96 h post-infection. Only a few cells were infected in siRNA stably expressing cells at 72 h post-infection, and at 120 h exhibited 45% fluorescence in siN2 stably expressing cells. When siRNA-expressing cells with CSFV infection were passaged at 72 h post-infection, almost all cells were infected at the second passage (data not shown). In order to study further the siRNA effect, the replication kinetics of CSFV was studied by real-time RT-PCR, and results showed that there was effective inhibition of virus replication. The RT-PCR used in this study showed that specific siRNAs were detected easily in siRNA stably expressing cells, suggesting efficiency and specificity of siRNA expression in siRNA stably expressing cells. For siS11, there was no detection of expression, yet it displayed a significant inhibition although it was not as efficient as siN1 or siN2. Considering that siS11 was below detection level, one could argue that the siS11 construct may be actually more effective than the two siN constructs.

Since RNAi is highly sequence-specific and it requires the target and targeting sequences to have 100% identity. *Pestiviruses* are genetically variable. The overall interspecies sequence identity within the genus is less than 70%, and sequence identity within a species varies from 75% to 100%. This genetic variety makes it impossible to design a siRNA able to cross-inhibit 3 species and very difficult to design one able to inhibit all isolates within the species. In order to evaluate the cross-inhibitory capabilities of our siRNAs, multiple alignments of *N^{pro}* and *NS4A* sequences of CSFV strains were conducted based on their availability in GenBank. Results showed that siN1, siN2 and siS11 could cover 71% (20/28), 60% (17/28) and 79% (22/28) of CSFV strains, respectively (data not shown), indicating that additional efforts should be undertaken to screen siRNAs with the widest cross-inhibitory effects on a range of CSFV strains.

Therapy of animal viral infections is still an interesting concept, although eradication is the worldwide policy, especially in developed countries, to prevent outbreaks of infectious diseases. Some studies so far have clearly shown a prolonged inhibition by siRNA; for example, retroviral mediated suppression of HIV transcription by a single siRNA has been observed for 1 year in a susceptible HeLa cell line (Yamagishi et al., 2009). This study has demonstrated that retroviral vector-mediated RNAi has potential for the treatment of CSFV infection. This method may not inhibit completely viral growth, but it merits further animal study to define its real therapeutic potential, and a replication-competent retroviral vector with siRNA in its genome can be used directly for treatment. Additionally, the combined use of different siRNAs might improve further the effect, and the siRNAs might be delivered to animals via transgenics (McGrew et al., 2004), or as a part of a vaccine potentially to create virus-resistant animal populations. The effect of siRNA can be combined use of different replication-competent retroviral vectors with siRNAs, and by repeated application. This concept might help reduce viral transmission during the immunity gap between vaccination and the onset of protection, thereby gaining precious time for bringing outbreaks under control.

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