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In vitro inhibition of CSFV replication by multiple siRNA expression

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ABSTRACT

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; however, RNA interference (RNAi) has been applied successfully to inhibit the replication of human and other animal viruses. In this study, three effective siRNAs targeting *NS3* of CSFV were selected. siNS3-2 targeting *NS3* gene was chosen for further experimentation, while siN1 and siN2 targeting N^{pro} gene, and siNS5B targeting *NS5B* gene describe previously. Single, double and quadruple anti-CSFV siRNA expression plasmids, with loxp sites at each end of the selectable marker genes, were constructed and analyzed using the same promoters or four different promoters, targeting N^{pro}, *NS3* and *NS5B* genes of CSFV. Results indicate that single or multiple siRNA expression plasmids can efficiently inhibit CSFV replication and that inhibition was markedly stronger when multiple siRNAs were expressed targeting different genes of CSFV. Since RNAi applied to anti-CSFV research, this study provides anti-CSFV methods by single and multiple siRNA expression which can target most viral isolates of different subtypes and prevent viral escape. It also provides a basis for development of CSFV-resistant transgenic pigs.

1. Introduction

RNA interference (RNAi) is a natural post-transcriptional gene silencing mechanism. It has been applied successfully to inhibit the replication of human and other animal viruses, such as human immunodeficiency virus type 1 (HIV-1) (ter Brake et al., 2008), SARS-CoV (Wu et al., 2005), influenza virus A (Sui et al., 2009), foot-and-mouth disease virus (Chen et al., 2004), porcine reproductive respiratory syndrome virus (Li et al., 2009a) and classical swine fever virus (Xu et al., 2008), both *in vitro* and *in vivo*.

Classical swine fever (CSF) is a highly contagious disease of pigs caused by infection with CSFV, and is a notifiable disease of the World Organization for Animal Health (OIE). CSF causes great economic losses in the pig industry worldwide. The disease was first recognized in China in the 1920s and there continue to be major epizootics in this country (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 of approximately 3400 amino acids. 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. N^{pro} is essential for evading the cellular antiviral defense system, and protects cells from double-stranded RNA-induced apoptosis (Ruggli et al., 2005). The *NS3* serine protease cleaves at downstream cleavage sites (Tautz et al., 1997; Tautz et al., 2000) and additionally possesses helicase and NTPase activities essential for viral RNA replication (Gu et al., 2000; Moulin et al., 2007). *NS5B*, an RNA-dependent RNA polymerase, is responsible for viral RNA replication (Zhang et al., 2005).

Previous studies of *in vitro* transcribed siRNA molecules (Xu et al., 2008) and retroviral-mediated RNAi (Li et al., 2010) showed that RNAi is capable of specific and efficient inhibition of CSFV replication. One problem is that siRNA cannot entirely inhibit CSFV replication. This has been suggested to be due to loss of siRNA activity caused by point mutation or deletion of the siRNA target site (Konishi et al., 2006; Westerhout et al., 2005). In this study, single, double, and quadruple anti-CSFV siRNA expression plasmids, with loxp sites at both ends of the selectable marker gene, were constructed and analyzed using the same promoters or four different promoters. Four different siRNA expressing from four different promoters can avoid recombination (ter Brake et al., 2008). Results show that CSFV inhibition was markedly stronger when multiple siRNAs were expressed targeting different genes of CSFV, and that siRNA target region mutation is not a major reason why CSFV replication cannot be completely blocked by RNAi. It also

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provides a basis for development of CSFV-resistant transgenic pigs, this is the reason why siRNA expression plasmids were constructed with loxp sites at each end of the selectable marker genes which could be excised by Cre-mediated loxP-flanked sequences.

2. Materials and methods

2.1. Cells, virus, and antiserum

Pig kidney cell line PK-15 (80 passages) and virulent CSFV strain Shimen were obtained from the Institute of Veterinary Drug Control, China. MAb WH303 (Kindly provided by Dr. Trevor Drew, Veterinary Laboratories Agency, Weybridge, UK), raised against CSFV strain UK/86/2 (Edwards and Sands, 1990; Paton et al., 1995) and with affinity for the E2 protein, was generated in the ascitic fluid of BALB/c mice.

2.2. Screening for siRNAs targeting NS3 gene

2.2.1. siRNA duplexes

All siRNAs were designed and synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China) with symmetrical 19-UU overhangs. The target sequences of siNS3-1, siNS3-2 and siNS3-3 were GCU UAU GAG UGG AAU ACA AUU (nucleotides 5713–5731 of CSFV genome, GenBank Accession AF092448), GAU GCC ACA ACC UAA GUU AUU (nucleotides 6046–6064), and CCA CUA UGA UCU ACU GCA AUU (nucleotides 6724–6742), respectively. A negative control siRNA was synthesized with the target sequence UUC UCC GAA CGU GUC ACG UUU, which has no matches either in the viral or the porcine genome.

2.2.2. siRNA transfection and CSFV infection

When cell monolayers were 50–70% confluent, siRNA was introduced using X-tremeGene siRNA Transfection Reagent (Roche, Indianapolis, IN) according to the Manufacturer's protocol, with a siRNA final concentration of 100 nM. The siRNA-transfected cells were infected with 2000 $CCID_{50}$ CSFV and cultured for 72 h at 37 °C. Indirect immunofluorescence assay (IFA), detection of the copy number of CSFV genomic RNA and detection of infectious virus production were performed as described previously (Li et al., 2010).

2.3. Plasmid construction

2.3.1. Single and double siRNA expression plasmid construction

Three 21nt candidate sequences, siN1, siN2 and siNS5B, designed and described previously (Xu et al., 2008), were chosen as siRNA for N^{pro} and *NS5B* genes. siNS3-2, described above, was chosen as siRNA for the *NS3* gene. Sequences selected for a negative siRNA control (siC) were the same as in Xu et al., 2008. Oligonucleotides were synthesized for each sequence by Nanjing Genscript Co., Ltd. (Nanjing, China; Table 1). These oligonucleotides were annealed and ligated to the *BamH1* and *HindIII* sites of pSilencer 3.1 H1 (Ambion, Austin, TX) to obtain plasmids pH1-(siRNAs), where "siRNAs" represents siN1, siN2, siNS3-2, siNS5B and siC.

H1-(siRNAs) were amplified by PCR using these plasmids as templates, with the following primers: H1-fw-n, H1-rev-n, H1-fw-c and H1-rev-c (Table 2). H1-(siRNAs) were digested and ligated to the appropriate insertion sites of PGKneotpAlox2 (Addgene, Plasmid 13444) to form plasmid pLox-(siRNAs) (see Fig. 2A–C). The insert sequences were confirmed by Shanghai Sangon Biological Engineering Technology & Service Co. (Shanghai, China).

2.3.2. siRNA4 expression plasmid construction

Construction of siRNA4 expression plasmid is based on pGenesil 1.1–1.4 (GeneSil Biotechnology Co., Wuhan, China), which respectively contain human U6, murine U6, human 7SK and human H1 polymerase III promoters (which were renamed phU6, pmU6, ph7SK and phH1). First, siRNA expression plasmids were constructed by inserting annealed oligonucleotides into the appropriate insertion sites, thereby encoding the siRNA transcription unit. Second, the pGenesil-siRNA4 plasmid was constructed from the different siRNA expression plasmids (phU6-siN1, pmU6-siNS3-2, ph7SK-siNS5B and phH1-siN2) by GeneSil Biotechnology Co. Finally, the siRNA cassette was digested and inserted into the *SalI*/*XhoI* sites of PGKneotpAlox2, yielding pLox-siRNA4 (see Fig. 2D).

2.3.3. Target site-EGFP fusion reporter plasmid construction

N^{pro} (nucleotides 347–877 of CSFV genome), *ΔNS3* (nucleotides 5885–6183) and *ΔNS5B* (nucleotides 10571–10912) were amplified by PCR using primers N^{pro}-fw, N^{pro}-rev, ΔNS3-fw, ΔNS3-rev, ΔNS5B-fw and ΔNS5B-rev (Table 2). The products were digested and ligated to the XhoI/PstI sites of pEGFP-N1 (Clontech, Mountain View, CA) to form plasmids pEGFP-N^{pro}, pEGFP-ΔNS3 and pEGFP-ΔNS5B.

2.4. Co-transfection of reporters and double siRNA or siRNA4 expression plasmids

PK-15 cells at 50–70% confluence in 96-well plates were co-transfected with 1 μg each of fusion reporter plasmid and double siRNA or siRNA4 expression plasmid, or control plasmid pLox-siC,

Table 1
List of siRNA sequences in this study.

Target name[a]	Sequences of siRNA (5′–3′)[b]
siN1 S	GACGGATCCGGATAGGTAGGGTGACAGGTTCAAGAGACCTGTCACCCTACCTATCCTTTTTTGGAAAAGCTTGCA
siN1 AS	TGCAAGCTTTTCCAAAAAAGGATAGGTAGGGTGACAGGTCTCTTGAACCTGTCACCCTACCTATCCGGATCCGTC
siN2 S	GACGGATCCGAACCCTGAAGTGGATTAGTTCAAGAGACTAATCCACTTCAGGGTTCTTTTTTGGAAAAGCTTGCA
siN2AS	TGCAAGCTTTTCCAAAAAAGAACCCTGAAGTGGATTAGTCTCTTGAACTAATCCACTTCAGGGTTCGGATCCGTC
siNS3-2 S	GACGGATCCGATGCCACAACCTAAGTTATTCAAGAGATAACTTAGGTTGTGGCATCTTTTTTGGAAAAGCTTGCA
siNS3-2 AS	TGCAAGCTTTTCCAAAAAAGATGCCACAACCTAAGTTATCTCTTGAATAACTTAGGTTGTGGCATCGGATCCGTC
siNS5B S	GACGGATCCGAATGAGAAGAGGGACGTCTTCAAGAGAGACGTCCCTCTTCTCATTCTTTTTTGGAAAAGCTTGCA
siNS5B AS	TGCAAGCTTTTCCAAAAAAGAATGAGAAGAGGGACGTCTCTCTTGAAGACGTCCCTCTTCTCATTCGGATCCGTC
siC S	GGAGGATCCGGACAGTGGGATGGATAGGTTCAAGAGACCTATCCATCCCACTGTCCTTTTTTGGAAAAGCTTCGG
siC AS	CCGAAGCTTTTCCAAAAAAGGACAGTGGGATGGATAGGTCTCTTGAACCTATCCATCCCACTGTCCGGATCCTCC

[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 (*BamHI*) and 3′ single-stranded overhangs (*HindIII*) 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).

Table 2
Primers used in this study.

Primer name	Sequences (5′–3′)	Enzyme sites
H1-fw-n	CTTGCGGCCGCGGTTTTCCCAGTCACGAC	NotI
H1-rev-n	GCGGCGGCCGCGCACCCCAGGCTTTACACTTT	NotI
H1-fw-c	CTTATCGATGGTTTTCCCAGTCACGAC	ClaI
H1-rev-c	GCGATCGATGCACCCCAGGCTTTACACTTT	ClaI
N^{pro}-fw	GGCCTCGAGATGGAGTTGAATCATTTTG	XhoI
N^{pro}-rev	GCCCTGCAGGCAACTGGTAACCCACAATG	PstI
ΔNS3-fw	GGCCTCGAGATGAGAGTCTTGGTCTTGATCCC	XhoI
ΔNS3-rev	GCCCTGCAGTGCGGTCATGGCTACTACCCG	PstI
ΔNS5B-fw	GGCCTCGAGATGGGTGCTGCTGGTTTCTTTGAA	XhoI
ΔNS5B-rev	GCCCTGCAGCCATTCCCTCTTTACTTTGTC	PstI
S-N^{pro}-fw	ATGGAGTTGAATCATTTTG	
S-N^{pro}-rev	GCAACTGGTAACCCACAATG	
S-ΔNS3-fw	AGAGTCTTGGTCTTGATCCC	
S-ΔNS3-rev	TGCGGTCATGGCTACTACCCG	
S-ΔNS5B-fw	GGTGCTGCTGGTTTCTTTGAA	
S-ΔNS5B-rev	CCATTCCCTCTTTACTTTGTC	

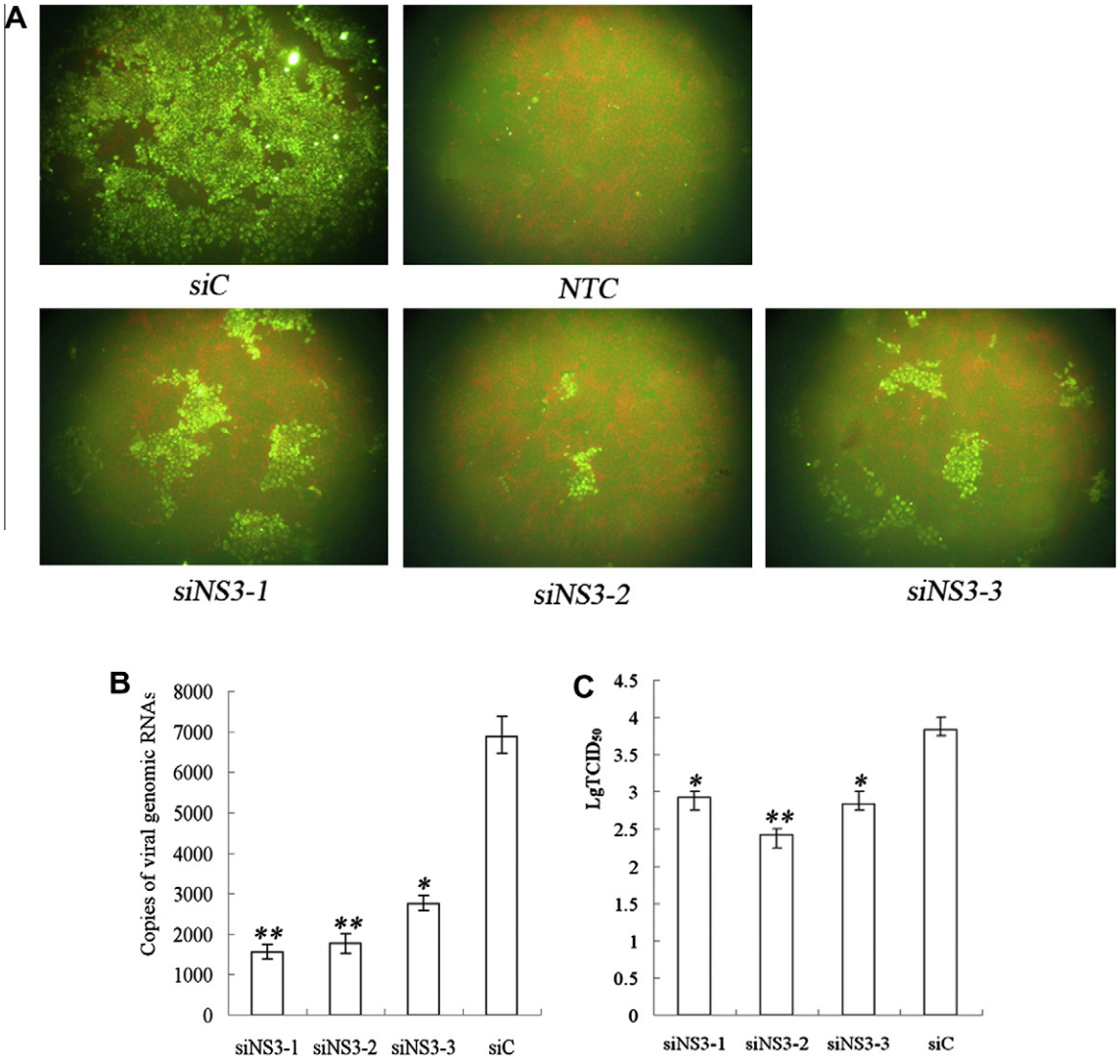

Fig. 1. Screening for siRNA targeting *NS3* gene. (A) Inhibition of CSFV replication by IFA in siRNA-treated cells (100× magnification). siC (siC transfection), NTC (mock transfection, stained with CSFV-negative serum), siNS3-1 (siNS3-1 transfection), siNS3-2 (siNS3-2 transfection), siNS3-3 (siNS3-3 transfection). (B) Reduction of viral genome replication in siRNA-treated cells. The CSFV genome copy numbers are the mean of three repeat experiments. (C) Inhibition of viable viral production in siRNA-treated cells. $CCID_{50}$ values are the means of three repeat titrations at the time points indicated. Ninety five percent confidence intervals are shown, $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$.

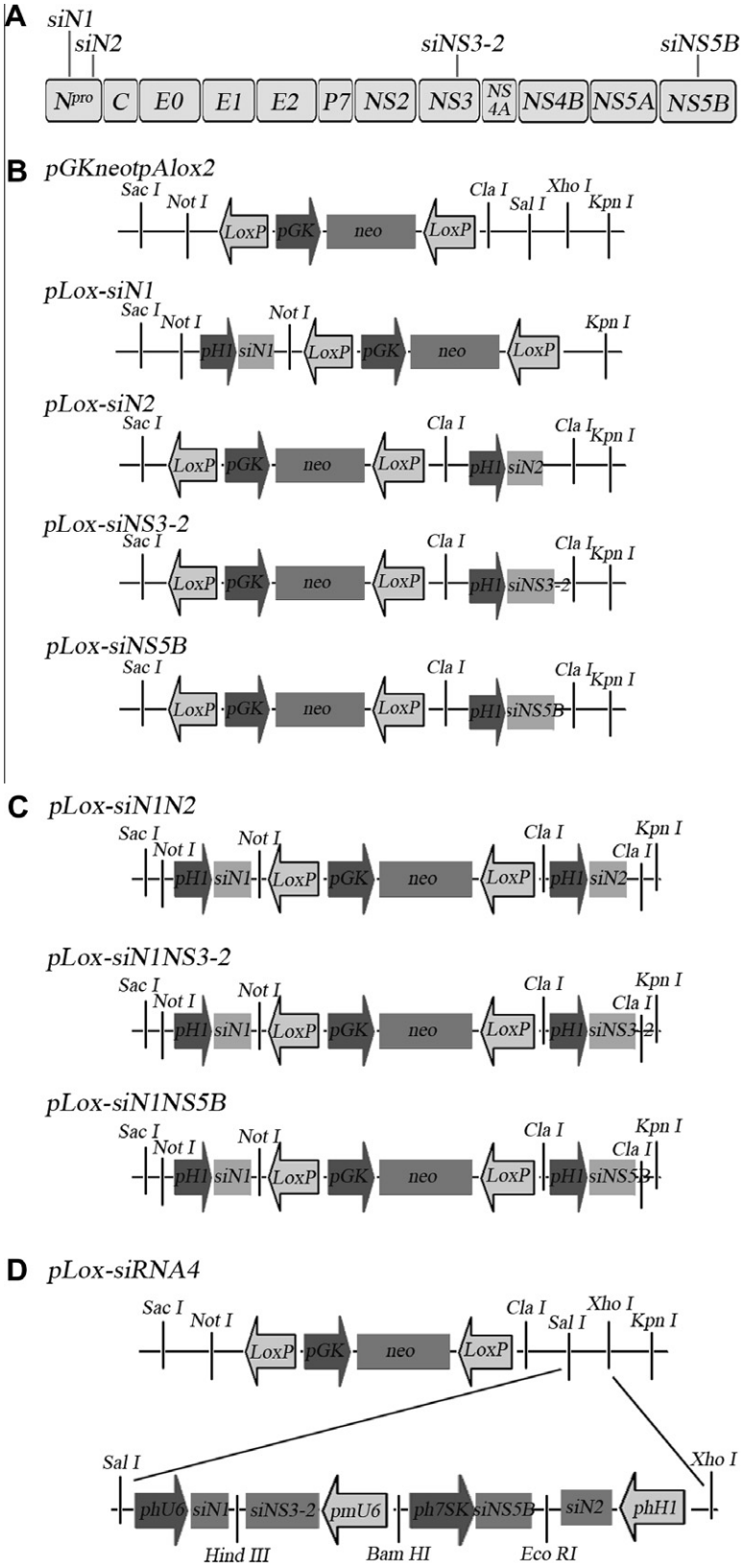

Fig. 2. Single or multiple siRNA expression plasmids construction. (A) Four siRNA sites targeting N^{pro}, *NS3*, *NS5B* genes of CSFV. (B) Single siRNA expression plasmid construction. (C) Double siRNA expression plasmid construction. (D) Four siRNA expression plasmid construction.

using FuGENE HD Transfection Reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. After 72 h, cells were examined and photographed with a Zeiss Axioskop 40 fluorescence microscope.

2.5. Single or multiple siRNA plasmid transfection and CSFV infection

PK-15 cells at 50–70% confluence in 12-well plates were transfected with 2 μg single or multiple siRNA plasmids using FuGENE

HD Transfection Reagent according to the Manufacturer's protocol, then infected with 1000 $CCID_{50}$ CSFV and cultured for 48 or 72 h. Indirect immunofluorescence assay (IFA), detection of CSFV genome copy number and detection of infectious virus production were performed as described previously (Li et al., 2010).

2.6. Analysis of CSFV escape RNA interference

pLox-siRNAs were linearized by KpnI and SacI and then used to transfect PK-15 cells. G418-resistant (500 μg/mL, GIBCO) cells derived from single cell cultures were screened for stable siRNA expression. Positive cells were examined by IFA for inhibition of CSFV replication after infection with CSFV.

Stable siRNA-expressing cells were used to seed 3 mL cell culture tubes (Nunc, Rochester, NY), incubated with 1000 $CCID_{50}$ CSFV in MEM for 2 h and then with MEM/2% FBS containing G418 (250 μg/mL) for 72 h. The tubes were then freeze–thawed three times, and the virus-containing supernatant was stored at −80 °C until further use. Using the above procedure, a total of 10 passages were carried out by transferring 1 mL of supernatant from the previous passage. The siRNA target sequences of passage 10 supernatants were amplified by PCR using primers listed in Table 2. Sequences were confirmed by Shanghai Sangon Biological Engineering Technology & Service Co. (Shanghai, China).

2.7. RNA purification

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) or QuickGene RNA cell culture cell HC kit S (FUJIFILM, Japan) according to the Manufacturers' protocols.

2.8. Statistical analysis

All numerical parameters, including genome copies and viral titers, are expressed as the mean ± 1 standard deviation. Student's *t*-test was used for the analysis of numeric parameters (SPSS 13.0 for Windows, SPCC Inc.) and calculation of *P* values.

3. Results

3.1. Screening for siRNA targeting the NS3 gene

To study the inhibitory effects of the three siRNAs targeting the *NS3* gene on CSFV replication, levels of viral antigen, viral genome copy number and production of viable virus in the cells with siRNA transfection and CSFV infection were examined. At 72 h post-CSFV infection, only a few cells displayed green fluorescence compared with the control (Fig. 1A). The viral genome copy number was significantly decreased in the cells receiving siNS3-1 ($P < 0.01$), siNS3-2 ($P < 0.01$) or siNS3-3 ($P < 0.05$) (Fig. 1B). The production of viable virus was significantly decreased in cells transfected with siNS3-1 ($P < 0.05$), siNS3-2 ($P < 0.01$) or siNS3-3 ($P < 0.05$) (Fig. 1C). These results indicate that the three siRNAs were effective inhibitors of CSFV replication, especially siNS3-2, which was therefore chosen for further experimentation.

3.2. Effect of multiple siRNA expression

PK-15 cell cultures were co-transfected with the multiple siRNA expression plasmids and EGFP reporters containing CSFV target sequences described above. EGFP expression levels were reduced only when the siRNA matched the reporter (data not shown). This confirmed that the observed inhibition of CSFV replication occurred through a sequence-specific mechanism. Similar results were obtained for multiple siRNA expression plasmids and matching reporters (Fig. 3). These data showed that each siRNA was still functional when two or four different siRNAs were expressed within a single vector.

3.3. Examination of the single or multiple siRNA effect by IFA

Using IFA and MAb WH303, levels of viral antigen were determined following CSFV infection of PK-15 cells transfected with single or multiple siRNA expression plasmids. At 72 h post-CSFV infection, 85% PK-15 cells expressing siC exhibited bright green fluorescence in the cytoplasm, indicating that most cells in the control were producing virus (Fig. 4I). By contrast, only a small proportion of cells (<5%) receiving single or multiple siRNA expression plasmids displayed green fluorescence, indicating that most cells were effectively protected by the siRNAs (Fig. 4A–H). Additionally, the level of protection was enhanced by multiple siRNA expression, especially that of siRNA4 targeting different genes of CSFV.

3.4. Single or multiple siRNA effects by real-time RT-PCR

Viral genome copy number was determined by real-time PCR (Mx3000P, Stratagene), using serially diluted plasmid pT-5′UTR as a standard. The R^2 value of the standard curve was 0.987 and the average amplification efficiency *E* was 0.994. Sequencing of products showed that they were the expected part of the 5′UTR of the gene, thereby demonstrating the specificity and reliability of the analysis. These data indicate that real-time PCR was highly reliable in the assay. The copy number of the viral genome was significantly decreased (77.2–96.8%) in cells expressing single or multiple siRNAs compared with siC cells ($P < 0.01$) (Fig. 5). The viral genome copy number was also significantly decreased in cells expressing siRNA4 compared with those expressing single siRNA plasmids ($P < 0.05$), indicating that inhibition efficiency was enhanced by multiple siRNAs targeting different genes of CSFV.

3.5. Single or multiple siRNA effects by infectious virus assay

As measured by $CCID_{50}$ assay, production of viable virus was decreased 2.9- to 30.6-fold in cells expressing single or multiple siRNAs compared with siC cells (Fig. 6). Production of viable virus was also significantly decreased in the siRNA4 cells expressing single siRNAs ($P < 0.05$), showing that multiple siRNA expression targeting different genes of CSFV provides more effective inhibition.

3.6. Genetic stability of CSFV exposed to RNA interference

To investigate if CSFV could develop resistance to RNAi, growth studies were carried out in stable siRNA-expressing PK-15 cells. Ten lines of cells were infected with CSFV and passaged 10 times. Viral genomes from each line at the 10th passage were sequenced in the siRNA target region and compared with the corresponding region of the parental virus genome. No mutations were detected (data not shown).

4. Discussion

CSFV genome is a single-stranded RNA that functions as both messenger RNA and a replication template, making it an attractive target for the study of RNA interference. Successful RNA interference with inhibition of CSFV replication has already been demonstrated by synthetic siRNA (Xu et al., 2008) and retroviral vector-mediated siRNA targeting N^{pro}, *NS4A* and *NS5B* genes (Li et al., 2010). To our knowledge, no siRNA targeting *NS3* gene of CSFV has been reported. *NS3* possesses serine protease, helicase and NTPase activities that are all essential for viral RNA replication.

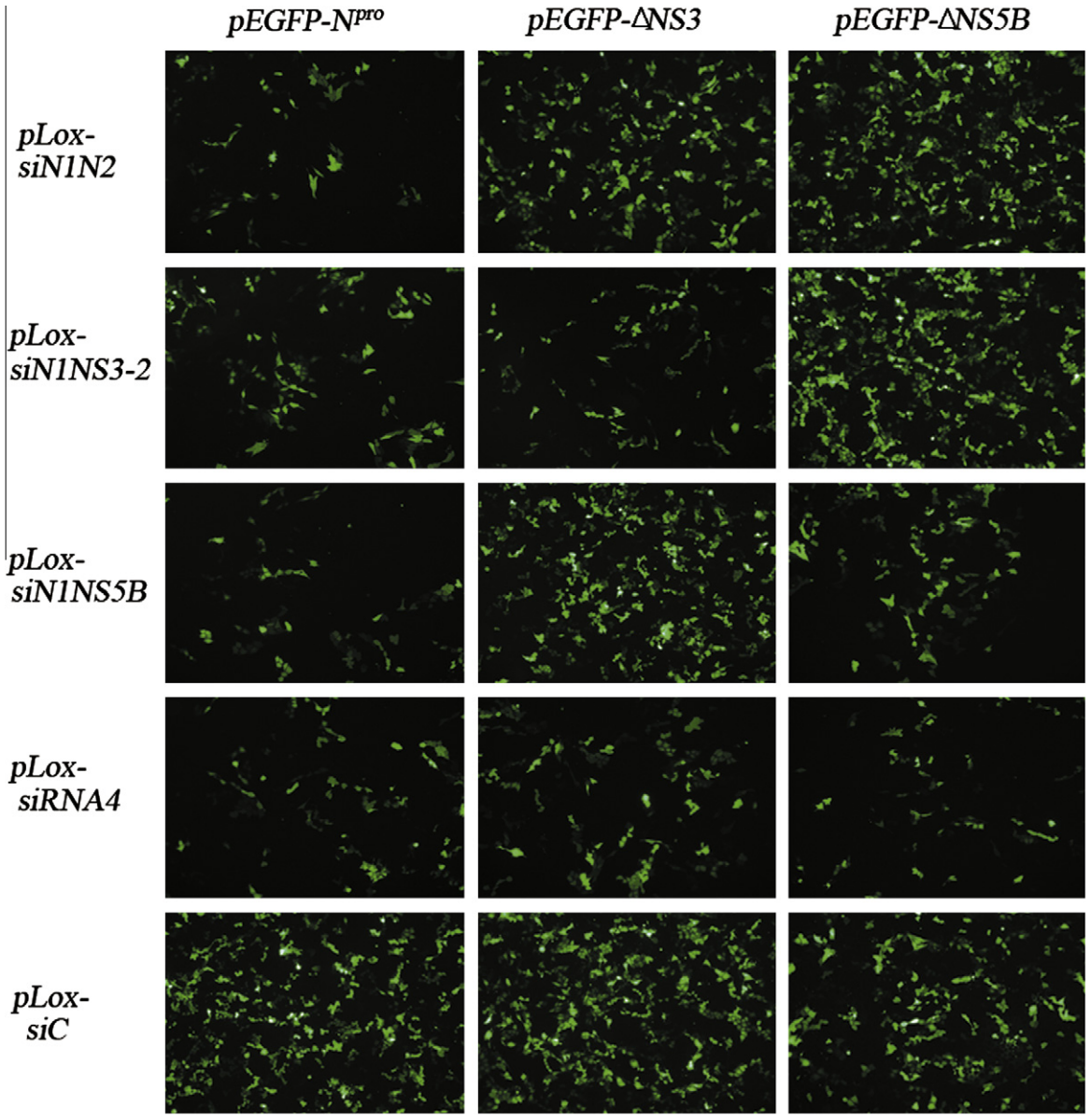

Fig. 3. Co-transfection of PK-15 cells with EGFP reporters and siRNA2 or siRNA4 expression plasmids (40× magnification).

Korf et al. (2007) reported inhibition of the HCV *NS3* gene by siRNA. In the present study, three siRNAs targeting the *NS3* gene of CSFV effectively inhibited CSFV replication, and siNS3-2 was most effective with a reduction in titer of up to 95.1%. These provided the candidate targeting sites to explore a new approach for preventing CSF.

For the development of an effective RNAi-based gene silencing against CSFV, a simple strategy would be to express multiple siRNA from a single or multiple promoters in one vector (Liu et al., 2008; Saayman et al., 2008; ter Brake et al., 2006; ter Brake et al., 2008). Previous studies have performed an analysis of a multiple siRNA expression strategy in which three or four siRNAs were expressed from the same promoters and found that this caused frequent recombinations at repeat sequences of the expression cassette, resulting in deletion of one or two cassettes (ter Brake et al., 2008). To prevent recombination, therefore, we selected four different promoters lacking sequence similarity for the four siRNA expression strategy. Human polymerase III promoters H1, U6 and 7SK and the murine polymerase III promoter U6 were selected because they have well defined transcription start and termination sites. Cloning the target sequences upstream of a reporter gene is a simple and convenient method for examining whether siRNA transcription occurs from multiple expression plasmids. Fig. 3 shows that each siRNA expression cassette was active, confirming that multiple siRNA expression from these promoters is a valid approach.

With vectors expressing siRNAs in PK-15 cells, multiple siRNA expression inhibited CSFV replication much more effectively than a single siRNA. This observation is consistent with a previous report that multiple siRNAs targeting the same mRNA results in enhanced gene silencing (Ji et al., 2003). Reduction in viral genome copy number and viable virus production at 48 and 72 h post-CSFV infection from multiple siRNA expression indicates that the effect of inhibition of CSFV replication is additive and maintained. This cumulative effect may restrict optimal shRNA processing by limiting the cellular components of the RNAi machinery (Castanotto et al., 2007), which might explain why each siRNA expression level from the siRNA4 was reduced compared to the single siRNA expression vector, as measured by Northern blot (ter Brake et al., 2008).

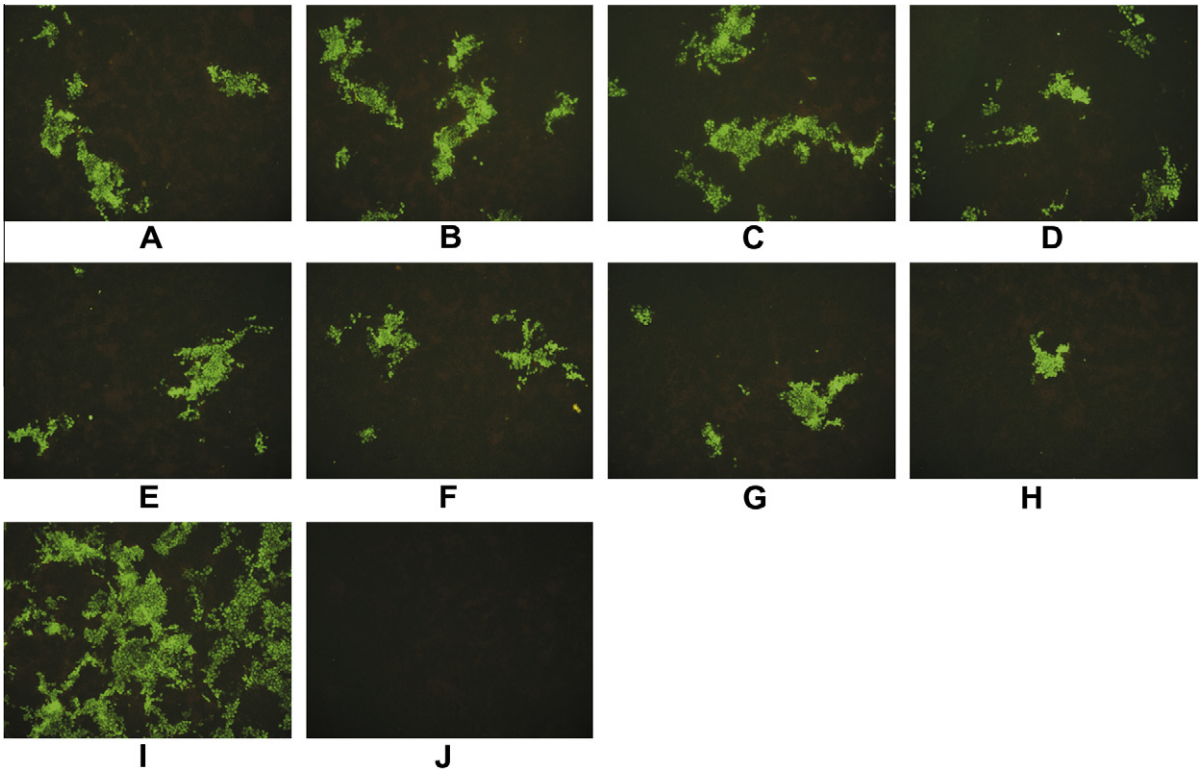

Fig. 4. Expression of viral antigen in PK-15 cells transfected with single or multiple siRNA expression plasmids at 72 h post-CSFV infection (40× magnification). (A) siN1 transfection, (B) siN2 transfection, (C) siNS3-2 transfection, (D) siNS5B transfection, (E) siN1N2 transfection, (F) siN1NS3-2 transfection, (G) siN1NS5B transfection, (H) siRNA4 transfection, (I) siC transfection, (J) NTC (mock transfection, stained with CSFV-negative serum).

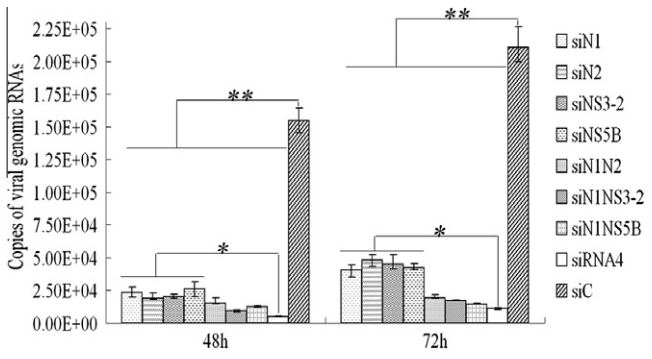

Fig. 5. Reduction of viral genome copy number in PK-15 cells transfected with single or multiple siRNA expression plasmids at 48 or 72 h post-CSFV infection. Genome copy numbers are the means of three experiments. The viral genome copy numbers per nanogram of total RNA from siC-transfected cells, calculated from the standard curve, were 1.55×10^5/ng and 2.11×10^5/ng at 48 and 72 h post-CSFV infection, respectively. Ninety five percent confidence intervals are shown, $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$.

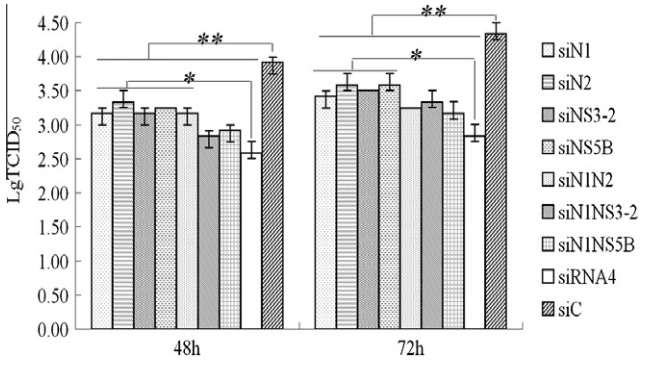

Fig. 6. Inhibition of viable viral production in PK-15 cells transfected with single or multiple siRNA expression plasmids at 48 or 72 h post-CSFV infection. $CCID_{50}$ values were calculated by the Kärber method. $CCID_{50}$ are the means of three repeat titrations at the time points indicated. The viable viral production from siC-transfected cells were $10^{3.92}$ $CCID_{50}$/mL and $10^{4.33}$ $CCID_{50}$/mL at 48 and 72 h post-CSFV infection, respectively. Ninety five percent confidence intervals are shown, $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$.

Viral replication is not completely blocked by the combined expression of multiple siRNAs. This is not surprising since RNAi does not target the incoming genome. Virus replication may still occur, therefore, albeit at a very low level, perhaps slowly accumulating until a certain threshold is reached.

Virus escape as a result of long-term exposure to antiviral RNAi has been observed in several studies (Das et al., 2004; Gitlin et al., 2002; Konishi et al., 2006). In the present study, however, no mutations of the target sites were detected even by the tenth virus passage in siRNA-expressing cells. These results indicate that target site mutation is not the major reason why CSFV replication cannot be completely blocked by RNAi.

The Cre/LoxP DNA recombinase system has been developed as a powerful tool for manipulating DNA both *in vitro* and *in vivo* (Guo et al., 1997; Li et al., 2009b; Schmidt et al., 2000), with many transgenic mouse models having been established using Cre/Loxp technology (Chen et al., 2008; Lin et al., 2008). Li et al. (2009b) created an EGFP reporter transgenic pig by applying the Cre/Loxp recombinase system. If the anti-CSFV activity of RNAi were to be combined with the technology of producing a transgenic pig, the result could be a pig resistant to CSFV infection. This is the reason why we constructed single and multiple anti-CSFV siRNA expression plasmids with loxp sites at each end of the selectable marker genes which

could be excised by Cre-mediated loxP-flanked sequences. Work to make this concept a reality is currently in progress.

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