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Cross-inhibition to heterologous foot-and-mouth disease virus infection induced by RNA interference targeting the conserved regions of viral genome

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Abstract

RNA interference (RNAi) is the process by which double-stranded RNA (dsRNA) directs sequence-specific degradation of messenger RNA in animal and plant cells. In mammalian cells, RNAi can be triggered by 21–23 nucleotide duplexes of small interfering RNA (siRNA). Strategies to inhibit RNA virus multiplication based on the use of siRNAs have to consider the high genetic polymorphism exhibited by this group of virus. Here we described a significant cross-inhibition of foot-and-mouth disease (FMD) virus (FMDV) replication in BHK-21 cells by siRNAs targeted to various conserved regions (5′NCR, VP4, VPg, POL, and 3′NCR) of the viral genome. The results showed that siRNAs generated in vitro by human recombinant dicer enzyme gave an inhibition of 10- to 1000-fold in virus yield of both homologous (HKN/2002) and heterologous (CHA/99) isolates of FMDV serotype O at 48 h post-infection (hpi). The inhibition extended to at least 6 days post-infection. For serotype Asia1, the virus yield in YNBS/58-infected cells examined at 12, 24, and 48 hpi decreased by ~10-fold in cells pretreated with HKN/2002-specific siRNAs, but there was no significant decrease at 60 hpi. The inhibition was specific to FMDV replication, as no reduction was observed in virus yield of pseudorabies virus, an unrelated virus. Moreover, we also demonstrated an enhanced viral suppression could be achieved in BHK-21 cells with siRNA transfection after an infection had been established. These results suggested that siRNAs directed to several conserved regions of the FMDV genome could inhibit FMDV replication in a cross-resistance manner, providing a strategy candidate to treat high genetic variability of FMDV.
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Keywords: FMDV; RNA interference (RNAi); Cross-inhibition; Conserved region; Viral genome

Introduction

RNA interference is an ancient natural antiviral mechanism that directs silencing of gene expression in a sequence-specific manner. It has already proven to be an invaluable research tool, allowing much more rapid characterization of the function of known genes. But can RNA interference be used as an effective antiviral strategy in mammalian cells? Recently, in tissue culture models, impressive results have been achieved against various cancer cells by using RNA interference to target oncogenes and against human immunodeficiency virus (HIV), influenza, and polioviruses by targeting viral genes (Coburn and Cullen, 2002; Damm-Welk et al., 2003; Ge et al., 2003; Gitlin et al., 2002). These findings raise our interests of

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whether RNAi can be an effective strategy against foot-and-mouth disease (FMD) virus (FMDV). Our previous study demonstrated that a DNA vector-based RNAi technology could specifically suppress the expression of FMDV VP1 in BHK-21 cells and inhibit FMDV replication in BHK-21 cells and suckling mice (Chen et al., 2004). Present data have generated much more hope for the use of RNAi as a novel antiviral therapy.

FMDV has the potential to cause explosive epidemics of FMD because of its diverse host range, low infectious dose requirement, high quantity of virus excretion, multiple routes of transmission, and short incubation period. The measures of control are routine vaccination and slaughter policy. As for routine vaccination, protective response usually occurs at 15 days after immunization. However, the speed of spread of FMDV is so rapid that pigs infected can release as much aerosol virus in a 24-h period as 3000 cattle. For slaughter policy, if an outbreak threatened to become extensive, it is considered that this strategy alone might not be sufficient to eradicate the virus. These situations demonstrate the importance of having a rapidly assimilated "emergency" vaccine capable of inducing early protection. The most important properties of an emergency FMD vaccine are the early induction of protective immunity and broad spectrum activity (Salt et al., 1998). Although the rapid induction of protection against aerosol infection within 4 days of vaccination has been an intensive area of study in cattle (Doel et al., 1994), sheep (Cox et al., 1999), and pigs (Salt et al., 1998), RNAi technology might have demonstrated more rapid speed of suppressing virus spread and more broader suppression spectrum by targeted to conserved regions of FMDV genome.

Like other RNA viruses, FMDV has high variability. Seven distinct serotypes and more than 60 subtypes of the virus have been characterized by serological analysis. FMDV is able to escape the host immune system by high variability of its surface antigens. Thus, establishing RNAi as a viable approach against FMDV requires resolving at least one major issue (Gitlin and Andino, 2003): the high genetic variability of viruses or viral escape, because viruses are likely to evade any given siRNA by mutations of the target sequences. Therefore, it may be important to produce multiple siRNAs, focusing on the conserved region of the viral genome or to use combinations of siRNAs (Gitlin and Andino, 2003; Gitlin et al., 2002; Song et al., 2003).

In this study, we describe a cross-inhibition of FMDV replication in BHK-21 cells by siRNAs targeted to various conserved regions of the FMDV genome. The multiple siRNAs have been used to target several viral genes that are essential for virus replication, including a long 5′ no-coding region (5′NCR), a shorter 3′ no-coding region (3′NCR), a small covalently bound virus-encoded protein (VPg), a viral polymerase (POL), and a viral capside protein (VP4). We further examine the possibility that the presence of intra-cellular target mRNA could sustain RNA silencing.

Results

siRNAs effectively suppressed the expression of fusion green fluorescent protein in BHK-21 cells

The siRNAs targeted to the conserved regions of FMDV genome were generated in vitro by human recombinant Dicer enzyme, as described in Fig. 1. To identify an effective inhibitory effect of siRNAs, the cDNA cassettes of these regions were inserted into the 5′ end of enhanced green fluorescent protein (EGFP) gene to construct reporter plasmids (Fig. 2). The reporter plasmids were cotransfected into BHK-21 cells with either the homologous siRNAs or the heterologous siRNAs. Results showed that the frequency of EGFP-expressing cell was markedly reduced in cells transfected with homologous siRNAs relative to cells transfected with heterologous siRNAs or non-transfected cells (Fig. 3A). Fluorescence-activated cell sorting demonstrated that the levels of inhibition mediated by the siRNAs were similar among the different experiment groups and significantly higher than the control group (cotransfection with heterologous siRNAs or without siRNAs) (Fig. 3B). To further demonstrate the levels of inhibition, cells were collected at 24 h post-transfection and real-time quantitative RT-PCR (Q-RT-PCR) analysis was performed. The level of target RNAs, as determined by RT-PCR, was also significantly reduced in cells transfected with homologous siRNAs (Fig. 3C). Melt-curve analysis confirmed the specific amplification of RT-PCR products (data not shown). Data of the real-time Q-RT-PCR were markedly reproducible based on the cycle threshold (CT) values derived from two separate experiments (Table 1). The results suggested that the siRNA generated by in vitro transcription could effectively and specifically inhibit the expression of FMDV conserved regions in BHK-21 cells.

Inhibition of FMDV replication in BHK-21 cells by siRNAs

To examine the suppression effect of siRNAs on the replication of virus, cells in one well of 96-well plates were transfected with 0.1 μg siRNAs. After 5 h, cells were infected with 100 50% tissue culture infective doses ($TCID_{50}$) of HKN/2002 (FMDV serotype O). Cells were observed continuously under the microscope. The results showed that cells transfected with siRNAs significantly reduced susceptibility to FMDV infection, relative to cells in the control groups (mock-transfection or infected with an unrelated pseudorabies virus (PRV) Ea). The effect of RNAi on viral replication was further investigated by measuring the $TCID_{50}$ of cells—lysed supernatant taken at 48 h and 96 h after viral challenge. Consistent with this observation, supernatant $TCID_{50}$ of FMDV was reduced by 50- to 100-fold (Fig. 4A), in comparison with the control group. Thus, siRNAs targeted to the conserved regions of FMDV genome were effective and specific, and resulted in an overall reduction of virus load. The ability of siRNA-transfected

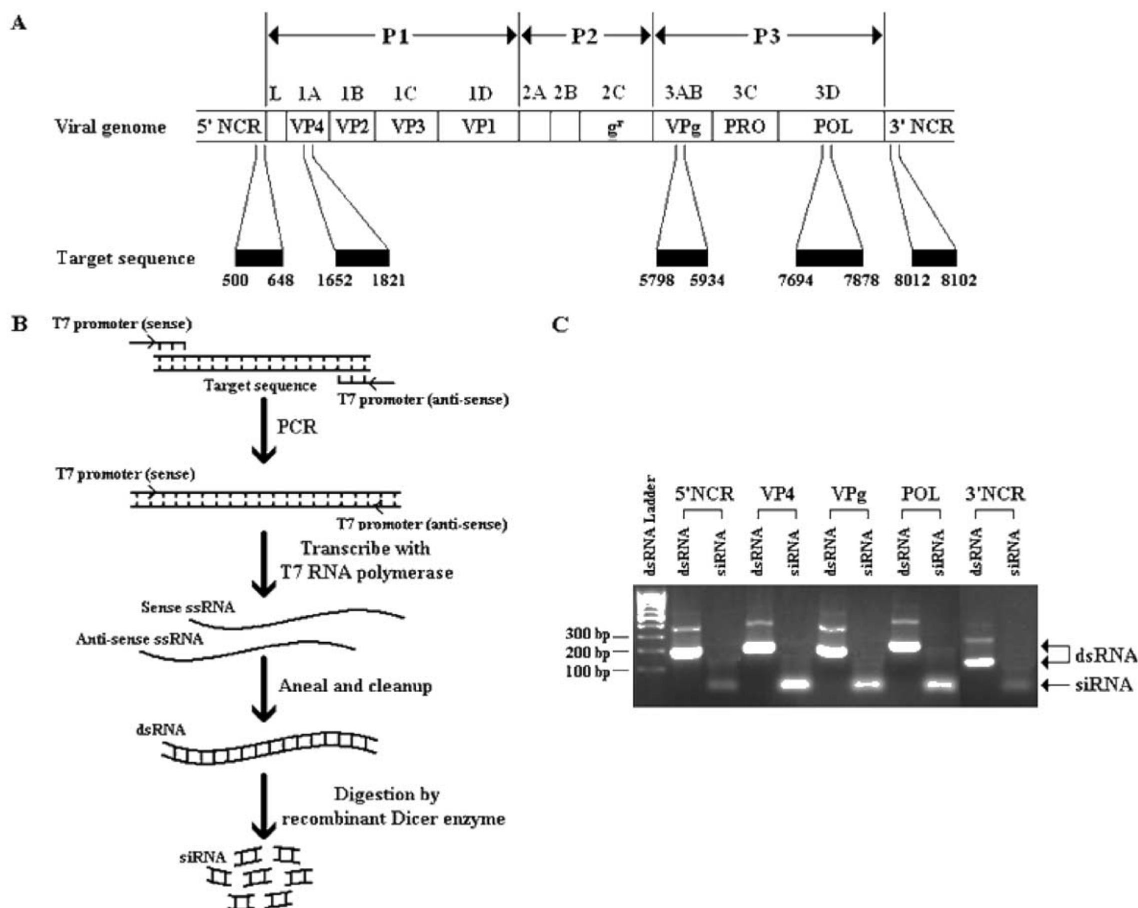

Fig. 1. Schematic representations of target viral mRNA and generation of siRNAs. (A) The FMDV genome contains a unique open reading frame. The filled boxes show the regions along viral genome targeted by FMDV-specific siRNAs. (B) The siRNAs are produced from in vitro transcription by T7 RNA polymerase and cleavage by recombinant Dicer enzyme. (C) The dsRNAs generated from in vitro strategy and the corresponding siRNAs were separated on a 2.5% agarose gel and visualized under UV light.

cells to resist virus infection was maintained over 6 days (data not shown).

To further test whether the siRNAs targeted to the conserved regions of FMDV genome could inhibit the replication of heterologous FMDV isolate, the CHA/99 strain of FMDV serotype O and the YNBS/58 strain of FMDV serotype Asia1 were employed to perform viral challenge. As described above, transfected cells (at 5-h post-transfection) were infected with 100 $TCID_{50}$ of CHA/99 or YNBS/58. A transient inhibition of virus yield was observed in cells transfected with FMDV-interfering siRNAs. The viral titer in CHA/99-infected cells was reduced by 15- to 1000-fold at 48 hpi (Fig. 4B). The antiviral effect extended to at least 6 days, as had been shown in cells infected by HKN/2002. Virus yield, in YNBS/58-infected cells examined at 12, 24, and 48 hpi, decreased by ~10-fold in cells pretreated with 5′NCR, POL, or 3′NCR siRNAs (Fig. 4C), but there was no significant decrease at 60 hpi. As expected, HKN/2002-specific VP4 and VPg siRNAs gave no marked inhibition to YNBS/58 at most of time points assayed. Mock transfection or cells infected with PRV did not show a significant reduction in virus yield (Fig. 4D). When cells were observed under the light-field microscope, the effect of siRNAs protecting cells from lysis was consistent with the reduction in virus yield (data not shown). These data indicated that transfection with siRNAs corresponding to conserved regions of FMDV genome not only conferred a clear reduction in homologous FMDV progeny, but also permitted the induction of cross-resistance to heterologous FMDV infection.

Enhanced RNAi effect by siRNA transfection after an established infection

Our previous study showed that RNAi effect could be enhanced by co-administration of the VP1 expression plasmid with siRNA into suckling mice before viral challenge. To further examine the hypothesis that RNAi effect may be long lasting if the target mRNA is continually available and to test if siRNAs can stably suppress FMDV in previously infected cells, BHK-21 cells were infected with 100 $TCID_{50}$ of FMDV before siRNA transfection. After 1 h, transfection was followed. Cells were observed continuously under the bright-field microscope at 24 h after transfection and supernatant of cells, used to measure viral titer, was collected at 48 h after infection. Except 3′NCR

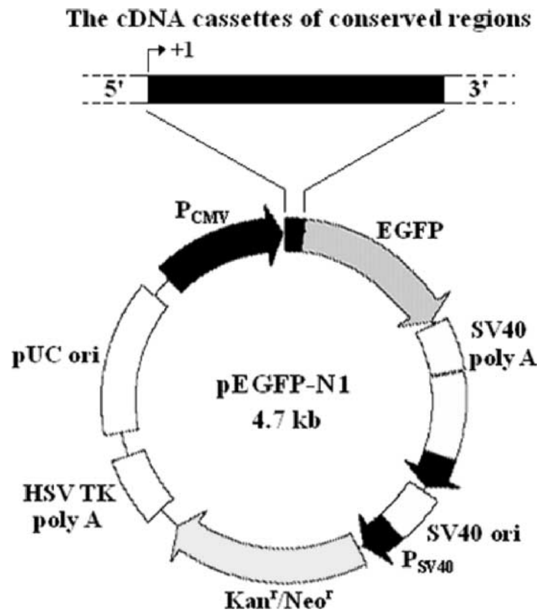

Fig. 2. Reporter plasmid construction. The cDNA cassettes corresponding to the conserved regions of the FMDV genome were cloned into the pEGFP-N1 vector. P_{CMV} = human cytomegalovirus (CMV) immediate early promoter; P_{SV40} = SV40 early promoter; EGFP = enhanced green fluorescent protein gene; Kan^r/Neo^r = kanamycin/neomycin resistance gene; SV40 poly A = SV40 early mRNA polyadenylation signal; HSV TK poly A = herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal; SV40 ori = SV40 origin of replication; pUC ori = pUC plasmid replication origin.

siRNAs, other four siRNAs in pre-infected cells were able to inhibit viral replication with a higher reduction in virus titer than that in pre-transfected cells (Student's t test, $P < 0.0005$ or $P < 0.01$) (Fig. 5A). Moreover, siRNAs effectively suppressed viral replication for prolonged periods in previously infected cells. The effects of suppression on virus replication were similar at 120 hpi by examining the cytopathic effect (CPE) under the microscope. However, FMDV suppression mediated by siRNA pre-transfection was progressively impaired and lost by 150 hpi. In contrast, viral inhibition by siRNAs in pre-infected cells was sustained for at least 198 hpi (Fig. 5B). This observation indicated that long-lasting and enhanced viral suppression could be achieved with siRNA in BHK-21 cells with an established infection, in agreement with previous studies (Song et al., 2003).

Discussion

Previously, we have investigated in vitro the inhibitory effect of VP1-specific siRNA on FMDV replication in BHK-21 cells (Chen et al., 2004). The results showed that BHK-21 cells transiently transfected with siRNA-expressing plasmids were specifically resistant to FMDV infection when exposed to 100 $TCID_{50}$ of virus with homologous VP1 sequence. However, the VP1-specific 21-nt siRNA with 2 mismatches over the heterologous virus did not confer significant inhibitory effect to viral replication of the heterologous virus. In this study, we demonstrated that siRNAs directed to the conserved regions of FMDV genome, namely 5′NCR, VP4, VPg, POL, and 3′NCR, could inhibit the replication of both FMDV strains serotype O (the homologous HKN/2002 and the heterologous CHA/99). Furthermore, the replication of FMDV serotype Asia1 (YNBS/58) could also be altered in cells transfected with some of HKN/2002-specific siRNAs. Our data suggest that it is feasible to produce multiple siRNAs, focusing on the conserved regions of the viral genome, against the high genetic variability of RNA viruses or the viral escape.

Five genes, 5′NCR, VP4, VPg, POL, and 3′NCR, were chosen as targets in this study for two reasons. Firstly, it is well known that different siRNAs induce different levels of RNAi activity (Hohjoh, 2002; Holen et al., 2002). Therefore, in order to realize efficient RNAi induction in mammalian cells, it would have been important to screen appropriate target regions along the FMDV genome conferring a strong RNAi activity. Secondly, because of the high genetic variability of FMDV, the relatively conserved regions with important functions were focused on. The 3′NCR is essential for FMDV replication in cell culture (Sáiz et al., 2001), and the evidence available for other picornaviruses indicates that 3′NCR is involved in the interaction with cellular and/or viral proteins during RNA replication (Mellits et al., 1998; Todd and Semler, 1996; Todd et al., 1995). The 5′NCR is predicted to form extensive secondary structures relative to translation initiation (Pilipenko et al., 1989). Because of the importance of 5′NCR and 3′NCR to FMDV infection, current development of anti-FMDV strategy has focused on the inhibition of viral infection by combinations of RNA transcripts corresponding to the 5′ and 3′NCR (Bigeriego et al., 1999). The RNA-dependent RNA polymerase (RdRp) 3Dpol and VPg of FMDV represent another two targets for the development of antiviral reagent since the initiation of picornaviral RNA replication is regulated predominantly by the interaction between 3Dpol, VPg, an RNA template, and other viral and cellular cofactors (Barton et al., 1995; Lama et al., 1994; Paul et al., 1998). 3Dpol is a key enzyme in viral RNA replication (Chen et al., 2003; O'Reilly and Kao, 1998; Steitz, 1998;). VPg has been shown to be essential for protein-primed initiation of both the positive and negative RNA strand replication (Cheney et al., 2003; Wimmer, 1982). VP4 is only one structural protein, located inside the mature virus particle, and involved in conversion of provirions to mature virions (Bachrach, 1968).

In this study, the efficiency of gene silencing varied between siRNAs targeted to different region of genome, and also varied between FMDV serotypes. The phenomenon is consistent with other investigations (Bohula et al., 2003; Harborth et al., 2001; Vickers et al., 2003). At present, there is still a lack of clear understanding on the mechanisms that determine the gene-silencing efficiency of a given siRNA. A number of hypotheses have been proposed in the literature,

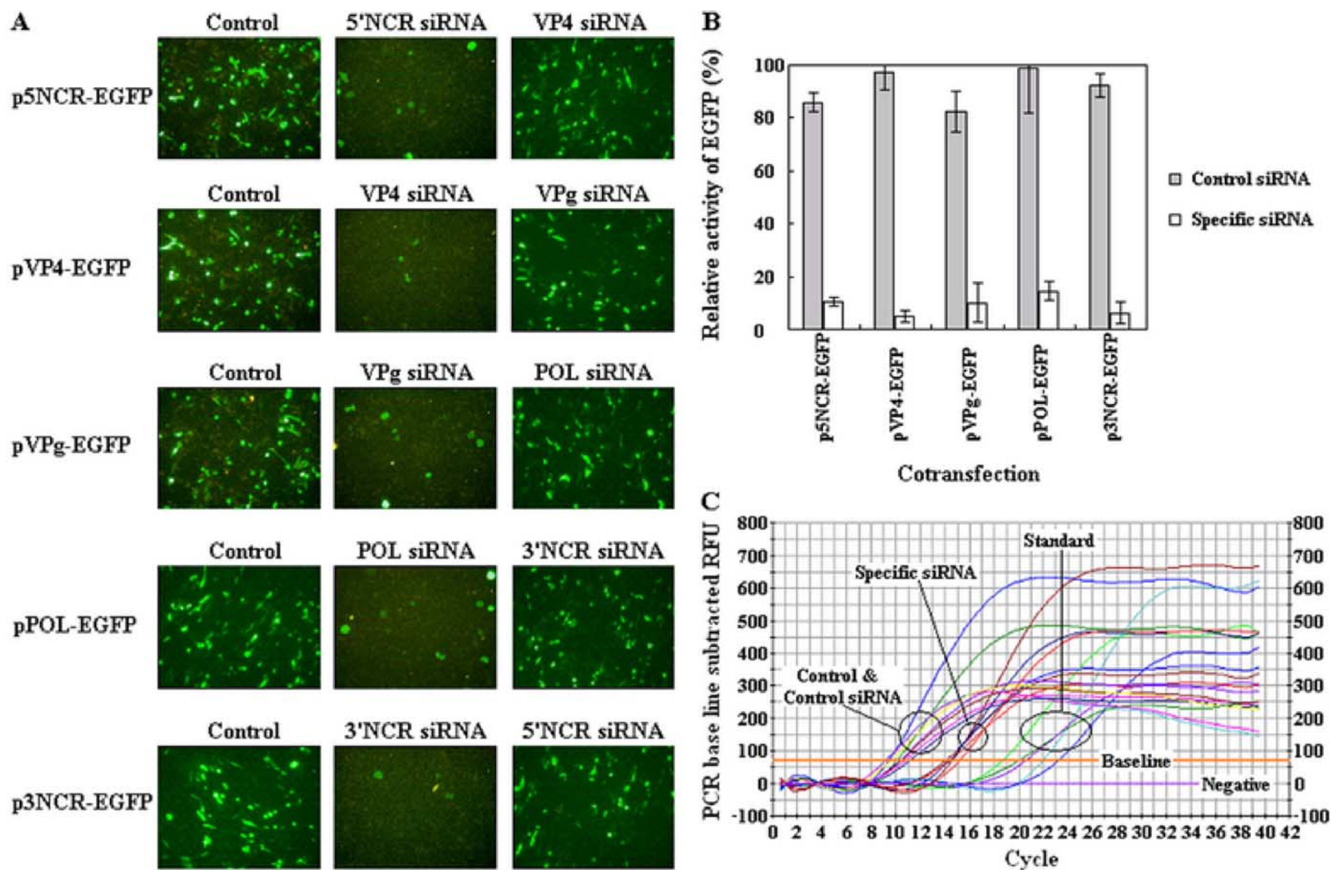

Fig. 3. Effect of RNAi on the expression of FMDV conserved region in BHK-21 cells. (A) Fluorescence micrographs of cells transfected with reporter plasmids and cotransfected with either the corresponding or non-corresponding siRNA with Lipofectamine 2000 regent (Invitrogen). At 24 h after transfection, representative fields were photographed. (B) Cells were analyzed for EGFP expression by fluorescence-activated cell sorting and the level of fluorescence relative to the control was quantitated. Data are the averages ± standard deviations from three separate experiments. (C) Real-time Q-RT-PCR analysis for expression of FMDV-conserved regions. One amplification plot of two separate real-time Q-RT-PCR (Table 1) of samples is shown here. The y-axis represents the PCR baseline subtracted relative fluorescence units (RFU). Cycle number is displayed on the x-axis.

including (a) local protein factors on the mRNA may cause the positional effect (Holen et al., 2002); (b) the local structure of the targeted mRNA may affect the accessibility of the siRNA (Bohula et al., 2003; Kretschmer-Kazemi Far and Sczakiel, 2003; Luo and Chang, 2004; Vickers et al., 2003); (c) factors such as sequence-dependent mRNA product release or differential efficiency of 5′ siRNA phosphorylation may influence the efficiency of the siRNA (Nykanen et al., 2001). Recent studies show that the gene-silencing efficiency of siRNA is strongly dependent on the local structure of mRNA at the targeted region. To further test the relationship between silencing efficiency and targeted mRNA, further work needs to be done. Additionally, in our study, the inhibition induced by siRNAs against the HKN/2002 sequences seems apparently somewhat greater against the heterologous isolate CHA/99 than the homologous virus for at least the 5′NCR target at 96 hpi (Fig. 4). 3′NCR siRNAs elicited more effective inhibitory response to HKN/2002 than to CHA/99, as expected previously. Besides the interfering factors described above as explanations, a relatively higher level of sequence similarity at the nucleotides for 5′NCR, VP4, VPg, and POL than that for 3′NCR might partially account for the unusual silencing efficiency against heterologous virus. Moreover, we could not rule out the possibility that the agreeable mismatches within siRNA duplexes enhance RNAi activity over the severe matches within

Table 1
Reproducibility of real-time Q-RT-PCR

Groups	Cycle threshold (CT) range	CT (mean)
p5NCR-EGFP	9.13 – 10.08	9.61
pVP4-EGFP	8.27 – 9.11	8.69
pVPg-EGFP	10.21 – 9.59	9.90
pPOL-EGFP	9.98 – 10.74	10.36
p3NCR-EGFP	8.92 – 10.43	9.68
p5NCR-EGFP + 5′NCR siRNA	13.56 – 14.62	14.09
pVP4-EGFP + VP4 siRNA	13.39 – 14.38	13.89
pVPg-EGFP + VPg siRNA	13.88 – 14.12	14.00
pPOL-EGFP + POL siRNA	13.48 – 14.43	13.96
p3NCR-EGFP + 3′NCR siRNA	15.65 – 15.20	15.43
p5NCR-EGFP + VP4 siRNA	9.06 – 9.43	9.25
pVP4-EGFP + VPg siRNA	8.57 – 9.18	8.88
pVPg-EGFP + POL siRNA	9.57 – 9.31	9.44
pPOL-EGFP + 3′NCR siRNA	11.11 – 11.14	11.13
p3NCR-EGFP + 5′NCR siRNA	9.54 – 10.32	9.93

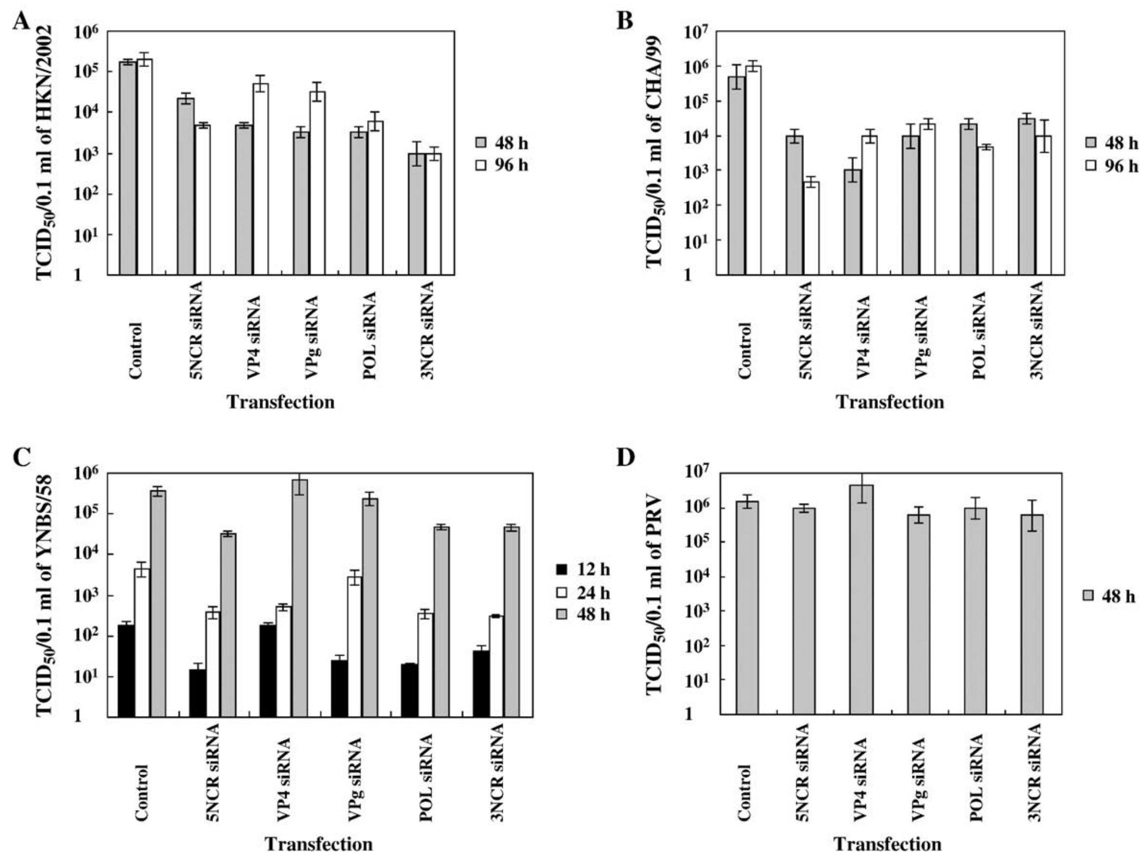

Fig. 4. Decrease of virus yield in BHK-21 cells transfected with FMDV-specific siRNAs. Cells were challenged with (A) FMDV HKN/2002, (B) FMDV CHA/99, and (C) FMDV YNBS/58 at 5 h post-transfection. Culture supernatants were collected at several time points assayed after FMDV challenge, and virus yields were measured by $TCID_{50}$. (D) As a control, an unrelated virus, PRV was used to examine the specific suppression activity of siRNA.

conventional siRNA elements in cultured mammalian cells (Hohjoh, 2004; Yu et al., 2002).

In agreement with the previous study (Song et al., 2003), our work also demonstrates an enhancement of RNAi effect on the replication of FMDV in BHK-21 cells after an infection has been established. However, it is not clear why siRNAs targeted to 3′NCR do not present an enhanced effect in this experiment. The possible reason is the severe specificity of 3′NCR mRNA in structure. Previous investigators have suggested that the presence of target mRNA may sustain siRNA (Platerk, 2002; Tuschl, 2002). We would like to attribute this promoted antiviral activity to the presence of a rudimentary form of immune memory (Gitlin and Andino, 2003). An alternative explanation is that the RNAi machinery could be naturally triggered in virally infected cells (Ding et al., 2004). When specific siRNAs generated in vitro are introduced into these cells, antiviral response based on RNAi will be faster, effective, and long-lasting. Additional experiments are clearly needed to explain this observation.

Our results take RNAi-based antiviral strategy a step forward to the control of FMD. Other than viral escape, several critical issues need to be addressed to improve RNAi effectiveness as an antiviral strategy against RNA viruses particularly in animal model systems and naturally susceptible animals, including persistence and enhancement of RNA silencing effect, siRNA delivery, systemic effect, etc. Researchers would have been expected to convert more and more new laboratory approaches based on RNAi to effective procedures to treat viral diseases.

Materials and methods

Cell culture and viruses

Baby hamster kidney cells 21 (BHK-21) were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 100 Uml^{-1} penicillin, and 100 μgml^{-1} streptomycin (Invitrogen). FMDV types O (HKN/2002 [GenBank accession number AF525458] and CHA/99 [unpublished]), FMDV types Asia1 (YNBS/58 [GenBank accession number AY390432]), and one PRV isolate (Ea [GenBank accession number AY318876]) were used for viral challenge.

Selection of target sequences

The reference sequences of the conserved regions of FMDV genome were obtained from the National Center for

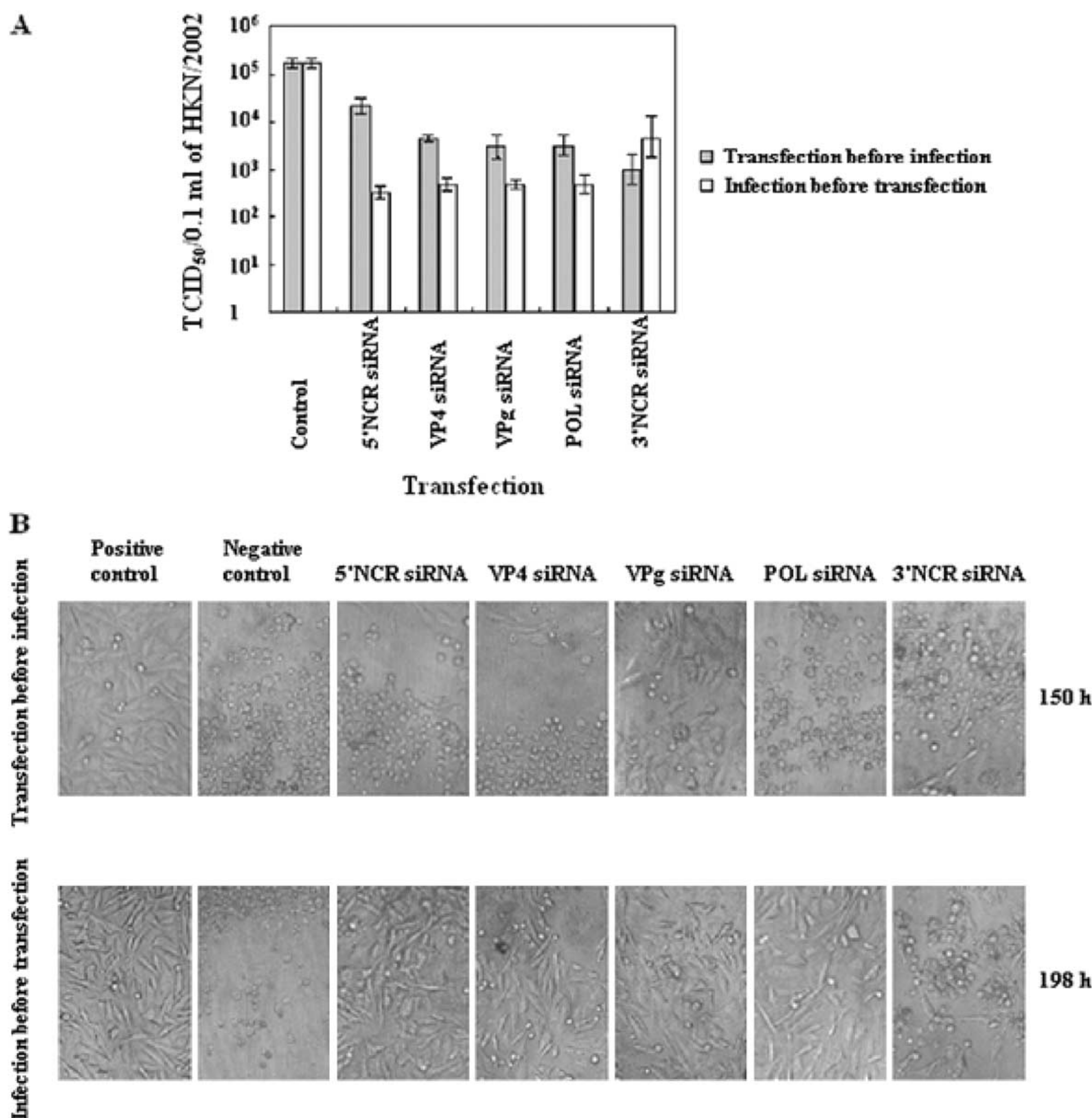

Fig. 5. The promoted antiviral effect by transfection with siRNAs after an infection has been established. (A) Cells were infected with FMDV HKN/2002 for 1 h prior to transfection with siRNAs. As a control, cells were transfected with siRNAs for 5 h prior to virus infection. Culture supernatants were collected at 48 hpi and the virus yields of two different treatments were measured. Error bars indicate standard deviations. Statistical analyses were carried out by Student's *t* test, indicating statistically significant difference ($P < 0.0005$ or $P < 0.01$) between the two different treatments and against the data of the control. (B) The promoted antiviral effects had been demonstrated till 198 hpi. Cells were visualized with an Olympus BH-2 microscope, and representative bright-field images were photographed. The cells on the upper panel were photographed at 150 hpi. The cells on the lower panel were photographed at 198 hpi.

Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) and compared with those of HKN/2002 by nucleotide (nt) BLAST. The genes and the regions of interest were essential during the life cycle of the virus and relatively conserved at the nucleotide sequences, as diagrammed in Fig. 1A. The region in 5′NCR contains the sequence corresponding to nt 500–648 of the genome of HKN/2002. The regions in VP4, VPg, POL, and 3′NCR contain the sequences corresponding to nt 1652–1821, 5798–5934, 7694–7878, and 8012–8102 of the genome of HKN/2002, respectively. These regions share significant sequence similarity at the nucleotides, ranging from 85% to 98% for serotype O, A, C, and Asia 1. Especially, the sequence similarity ranges from 91% to 98% between the serotype O HKN/2002 and CHA/99, namely, 95%, 94%, 97%, 98%, and 91% for 5′NCR, VP4, VPg, POL, and 3′NCR, respectively. For the serotype O HKN/2002 and serotype Asia1 YNBS/58, the regions in 5′NCR, POL, and 3′NCR show similarities of 93%, 97%, and 91%, respectively, but those in VP4 and VPg show no significant sequence similarity.

Preparations of siRNAs

The siRNAs were produced from in vitro transcripts according to the Dicer siRNA Generation Kit (Catalog Number T510001) purchased from Gene Therapy Systems,

Inc. The Dicer siRNA Generation Kit mimics the natural RNA interference process by using recombinant human dicer enzyme to cleave in vitro-transcribed dsRNA into a pool of 22-bp siRNAs (Fig. 1B). The total RNA of HKN/2002 genome was prepared with Trizol reagent (GIBCO BRL) according to the manufacturer's instructions, and the cDNAs of five targeted regions were obtained by reverse transcription-PCR (RT-PCR) amplification with superscript one-step RT-PCR system (GIBCO BRL) using the primers as followed: for 5′NCR, 5′-GGAATTCATGTTTGC-CCGTTTTCATGAG-3′ (sense) and 5′-CGGGATCC CCTCTAGACCTGGAAAGA-3′ (antisense); for VP4, 5′-CGGGATCC TTGTTCTGAGTGTTGGTTG-3′ (sense) and 5′-GGGGTACCTTGTTCTGAGTGTTGGTTG-3′ (antisense); for VPg, 5′-GGAATTCATGAAGCTCCCA-CAGCAGGAG-3′ (sense) and 5′-CGGGATCCTTC-ACTTTCAAAGCGACA-3′ (antisense); for POL, 5′-GG-AATTCATGCCAGCTGACAAAAGCGAC-3′ (sense) and 5′-CGGGATCCACGGAGATCAACTTCTC-3′ (antisense); for 3′NCR, 5′-GGAATTCATGTCCCTCAGATGTCA-CAATTG-3′ (sense) and 5′-CGGGATCCAAATAGGAA-GCGGGAAAAA-3′ (antisense). The cDNA sequences were determined by sequencing. We further obtained the in vitro RNA transcription template by adding T7 RNA polymerase promoters to the cDNAs by standard PCR. The gene-specific PCR primers containing T7 promoter sequence (5′-GCGTAATACGACTCACTATAGGGAGA-3′) were derived from the primers for RT-PCR described above. Then the generation, recovery, and quantitation of dsRNAs were performed with the Turboscript T7 Transcription kit. Finally, the siRNAs generated by Recombinant Dicer Enzyme were purified with RNA purification column to remove salts, unincorporated nucleotides, and the undigested dsRNA. The purified siRNAs were qualified and stored to be used in subsequent transfection experiments (Fig. 1C).

Construction of reporter plasmids

To analyze the silencing of the expression of exogenous reporter gene, five reporter plasmids were constructed by cloning the cDNAs of five target sequences of FMDV genome into the *Sal*I–*Bam*HI sites of pEGFP-N1 (Clontech, Palo Alto, CA) to form fusion green fluorescent proteins, respectively (Fig. 2). The resultant plasmids were p5NCR-EGFP, pVP4-EGFP, pVPg-EGFP, pPOL-EGFP, and p3NCR-EGFP. The correct open reading frames confirmed by sequencing retained the fluorescent properties of the fusion protein.

Transient cellular transfection and analysis of the targeted gene and EGFP expression in BHK-21 cells

Cell cultures were incubated at 37 °C with 5% CO_2. The day before transfection, cells were trypsinized, diluted with fresh medium, and seeded into 96-well culture plates (approximately 0.5 × 10^{-5} cells/well). BHK-21 cells (about 80% confluent) were transfected with Lipofectamine 2000 reagent (Invitrogen) by the manufacturer's protocol in the presence of 0.2 μg recombinant reporter plasmid and/or 0.1 μg siRNA. After an additional 24 h of incubation, cells were observed for the expression of green fluorescent protein on an Olympus BH-2 microscope and photographed using a Nikon E950 video camera at a magnification of ×40 with an exposure time of 1/8 s. Cells were further subjected to fluorescence-activated cell sorting.

For detection of the targeted gene expression in BHK-21 at 24 h after transfection, total RNA was extracted from BHK-21 culture with Trizol reagent, incubated for 1 h at 37 °C with Dnase RQ1, and subjected to the real-time Q-RT-PCR analysis. Briefly, the real-time Q-RT-PCR was performed in a 96-well plate (BioRad, Hercules, CA) in a 20-μl reaction volume containing components of the SYBR RT-PCR Kit (Perfect Real Time) (TaKaRa, Kyoto, JP). The 20-μl reaction mixture contained 10 μl SYBR master mix (2×), 0.4 μl of 0.2 μM forward primer, 0.4 μl of 0.2 μM reverse primer, 2.0 μl of 1 μg RNA sample, and 7.2 μl of water. The RT-PCR thermocycling program consisted of 50 °C for 30 min, 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The primers employed were the same as the primers for RT-PCR described above. To confirm the specific amplification, melt-curve analysis of the RT-PCR products was performed according to the manufacturer's protocol. Fluorescence was measured following each cycle and displayed graphically by iCycler iQ Real-Time PCR Detection System Software Version 3.0A (BioRad, Hercules, CA). RT-PCR products were further cloned into T-vector for sequencing.

Viral challenge assay in BHK-21 cell

Virus infectivity was determined by serial dilution of sample on BHK-21 cells grown in 96-well plates and the virus titer was calculated as a $TCID_{50}$ by the Reed–Muench method (Reed and Muench, 1938). A viral suspension titrated at 10^5 to 10^7 $TCID_{50}$ per 0.1 ml was used for viral challenge. BHK-21 cells (about 80% confluent) grown in 96-well plates were transiently transfected with 0.1 μg siRNA per well. After 5 h of transfection, the transfection complex was removed and cells were washed twice with RPMI 1640 medium. The transfected cells in one well of the 96-well plates were then infected with 100 $TCID_{50}$ of virus per 0.1 ml. After 1 h of adsorption, the inoculum was removed and the cells were washed twice with RPMI 1640 medium. The infection then proceeded in RPMI 1640 medium supplemented with 10% fetal bovine serum. For detecting the therapeutic potential of siRNA, in another parallel experiment, transfection was performed after 1 h of infection with the virus. FMDV replication in BHK-21 cells was evaluated by virus titer ($TCID_{50}$) in supernatant taken at the time indicated.

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