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Transient inhibition of foot-and-mouth disease virus replication by siRNAs silencing VP1 protein coding region

Ke Lv, Yingjun Guo, Yiliang Zhang, Kaiyu Wang, Ka Li, Yan Zhu, Shuhan Sun *

Department of Medical Genetics, Second Military Medical University, 800 XiangYin Road, Shanghai 200433, PR China

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ABSTRACT

Foot-and-mouth disease virus (FMDV) is the causative agent of foot-and-mouth disease, a severe, clinically acute, vesicular disease of cloven-hoofed animals. RNA interference (RNAi) is a mechanism for silencing gene expression post-transcriptionally that is being exploited as a rapid antiviral strategy. To identify efficacious small interfering RNAs (siRNAs) to inhibit the replication of FMDV, candidate siRNAs corresponding to FMDV VP1 gene were designed and synthesized in vitro using T7 RNA polymerase. In reporter assays, five siRNAs showed significant sequence-specific silencing effects on the expression of VP1-EGFP fusion protein from plasmid pVP1-EGFP-N1, which was cotransfected with siRNA into 293T cells. Furthermore, using RT-qPCR, viral titration and viability assay, we identified VP1-siRNA517, VP1-siRNA113 and VP1-siRNA519 that transiently acted as potent inhibitors of FMDV replication when BHK-21 cells were infected with FMDV. In addition, variations within multiple regions of the quasispecies of FMDV were retrospectively revealed by sequencing of FMDV genes, and a single nucleotide substitution was identified as the main factor in resistance to RNAi. Our data demonstrated that the three siRNA molecules synthesized with T7 RNA polymerase could have transient inhibitory effects on the replication of FMDV.

1. Introduction

Foot-and-mouth disease (FMD) is a disease of cloven-hoofed animals such as cattle, pigs, sheep and goats, the pathology of which causes in adult animals, signs of fever, vesicles in the mouth, feet and udders, loss of milk production and also causes death in young animals (Haydon et al., 2004). The impacts of a regional or nationwide outbreak of FMD can pose severe economic problems, such as loss in the United Kingdom, Netherland and Taiwan (Chen et al., 2007; Saiz et al., 2002; Shih et al., 1998; Thompson et al., 2001).

Conventional vaccines have potential to control major epidemics of FMD and new generation vaccines, based on viral proteins, protein fragments and nucleic acids, each have promising future in terms of their own advantages over the existing conventional vaccines. Nevertheless, vaccination still remains contentious owing to its limitations. For example, conventional inactivated virus vaccines may leave residual live virus in vaccines, DNA vaccines elicit inadequate neutralizing antibody to protect against the disease in swine (Balamurugan et al., 2004; Ward et al., 1997), chimeric vaccines have possibilities to lead to production of virulent viruses and to a lack of thermostability for inactivated vaccine production (Van Rensburg and Mason, 2002) and so on. Furthermore, it is imperative to use a new antiviral strategy to compensate vaccine's requirement of at least 7 days to induce protection while the clinical disease and infectivity appear in infected animals as early as 2–3 days post-exposure (Grubman and de los Santos, 2005).

Since the discoveries of dsRNA-induced gene-silencing in plants (Napoli et al., 1990) and *Neurosporo crassa* (Cogoni and Macino, 1997) and the decisive unraveling of the mechanisms underling RNAi phenomenon in the worm *Caenorhabditis elegans* (Fire et al., 1998), RNAi has been used as a powerful laboratory antiviral tool and may offer a new therapeutic option for viral infections, such as human immunodeficiency virus (Bennasser et al., 2007; Nekhai and Jerebtsova, 2006), hepatitis B and C virus (Kim et al., 2006; Radhakrishnan et al., 2004; Rendall, 2005) and SARS-CoV (Akerstrom et al., 2007; Wu et al., 2005), as well as a valuable tool to study endogenous genes function. Recently, several laboratories used RNAi to inhibit foot-and-mouth virus infection in cell culture or in animals taking forms of chemically synthesized siRNA, plasmid encoding shRNA, and adenovirus encoding shRNA (Chen et al., 2004, 2006; de los Santos et al., 2005; Kahana et al., 2004; Mohapatra et al., 2005), showing rapid decrease of FMDV replication.

FMDV, has a positive-strand RNA of approximately 8500 nucleotides in length and consists of only a single long open reading frame (ORF), is a member of the Picornaviridae family (Belsham, 2005; Mason et al., 2003). It is known that the viral genomic RNA in positive-stranded picornaviruses was used both as a

* Corresponding author. Tel./fax: +86 21 25070331.
E-mail address: shsun@vip.sina.com (S. Sun).

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messenger for translation and as a template for negative-strand synthesis and there might be a "switch" between these processes (Belsham, 2005; van Rij and Andino, 2006). Recent studies of poliovirus (Gitlin et al., 2005) and influenza virus (Kahana et al., 2004) showed that intriguingly only the positive RNA strand was targeted by RNAi. As the cleavage at a specific location might result in degradation of the full length of virus, we chose VP1 of FMDV as a specific target to inhibit the replication of FMDV, which encodes a structural protein and contains an Arg–Gly–Asp (RGD) tripeptide sequence in G-H loop responsible for binding of fibronectin to its cellular receptor integrins (Mason et al., 2003).

In this work, we made a number of small interfering RNAs (siRNAs) targeting the VP1 gene of FMDV which were quickly and efficiently synthesized in vitro using T7 RNA Polymerase. We tested and identified multiple effective siRNAs candidates by confirming sequence-specific inhibition in independent enhanced green fluorescence protein (EGFP) reporter assays. Furthermore, three of these siRNAs were validated to transiently inhibit FMDV replication in BHK-21 cells. Additionally, we demonstrated that viral populations consisting of variant spectra might be the obstacles affecting the siRNAs inhibition in cell culture.

2. Materials and methods

2.1. In vitro synthesis of siRNAs

siRNAs were generated using the T7 RiboMAX Express RNAi system (Promega, Madison, WI, USA). In brief, sequence candidates from VP1 gene of FMDV for siRNAs synthesis were selected using the web-based tool siRNA Target Designer (Version 1.51) from Promega (http://www.promega.com/siRNADesigner/) which meets the sequence requirement that the presence of a "5′-GN_{17}C-3′" sequence in the target RNA is needed. Specificity of the sequence candidates was verified by BLAST search (http://www.ncbi.nlm.nih.gov/blast/). The DNA templates for in vitro transcription of siRNAs contain T7 RNA polymerase promoter sequence and six extra nucleotides upstream of the minimal promoter sequence and an additional two adenine nucleotides are added to the 5′ end of templates. The pEGFP-N1-siRNA (Qin et al., 2004) and scrambled-siRNA were also chosen as positive and negative siRNA control, respectively. The oligonucleotide-directed production of small interfering RNA with T7 RNA polymerase was carried out using T7 RiboMAX Express RNAi system (Betz, 2003) according to the manufacturer's instructions (Promega, Madison, WI, USA). Sense and antisense 21-nucleotide RNAs generated in separate reactions were annealed by mixing both transcription reactions, incubating at 70° for 10 min, followed by 20 min at room temperature to obtain small interfering double-stranded RNA synthesized by T7 RNA polymerase. The mixture was then purified by isopropanol precipitation followed by 70% ethanol washing, dried and resuspended in appropriate amount of nuclease-free water. siRNA aliquots were stored at −80° until used.

2.2. Cell culture and FMDV titration

Human embryonic kidney cell line (HEK) 293T cells and baby hamster kidney cell line (BHK-21) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO™, Invitrogen Corporation, Grand Island, NY, USA) supplemented with 5% heat-inactivated calf serum at 37° in a humidified atmosphere of 5% CO_2.

The virus used for infection was FMDV O isolate O/NY00 (GenBank Accession No. **AY333431**), which was kindly provided by Prof. Jin Ningyi (Dept. Of Virology, The Military Veterinary Institute, Quartermaster University of PLA, Changchun, Jilin), and its growth, isolation and titration were all conducted using BHK-21 cells. The titration was performed as follows. BHK-21 cells were seeded into 96-well plates 1 day before infection. Supernatant from viral infected samples were 10-fold serially diluted and added to wells, 25 μl per well in 8 duplicate. Four days after infection, the 50% tissue culture infective doses ($TCID_{50}$) were calculated using the Reed–Muench formula (Reed and Muench, 1938).

2.3. Generation of reporter plasmids

The recombinant plasmids pVP1-EGFP-N1 containing the whole length of VP1 gene sequence and p3C-EGFP-N1 containing 3C gene sequence were constructed as follows. FMDV (NY00) genomic RNA was extracted from samples of FMDV-infected BHK-21($TCID_{50}$ = 10^7/ml), which had been subjected to three cycles of freezing and thawing, according to the manufacturer's instructions (Body fluid viral DNA/viral RNA mini-prep Kit, V-gene Biotechnology Ltd., PR China). Then the viral RNA were reverse transcribed to cDNA using ExScript™RT Reagent Kit (Perfect Real Time) (TaKaRa) according to the manufacturer's manual. The VP1 fragment (639 bp) of FMDV were amplified with a sense EcoRI-adapter primer (5′-CCGGAATTCATGACCACCTCCACAGG-3′) and an antisense BamHI-adapter primer (5′-CGCGGATCCTCCAAAAGCTGTTTCACAG-3′), while the 3C (639 bp) with a sense EcoRI-adapter primer (5′-CCGGAATTCATGAGTGGTGCTCCCCCG-3′) and an antisense BamHI-adapter primer (5′-CGCGGATCCTCCTCGTGGTGTGGTTCG-3′), using Pfu MasterMix (Tiangen, Biotechnology Co. Ltd., Beijing, PR China). The PCR products were then cloned into the unique site of EcoRI and BamHI of pEGFP-N1 vector (Clontech, Palo Alto, CA) upstream of the EGFP gene. The sequences of the inserts were checked by restriction enzyme digestion and the DNA sequencing.

A reporter plasmid, designated pVP1-EGFP-N1-410A, was generated by utilizing the pVP1-EGFP-N1 plasmid as template, and a G → A mutation was generated at the position of 410 of the pVP1-EGFP-N1, which is equivalent to the VP1-410 G → A variations using the QuikChange site-directed mutagenesis kit (Stratagene). Briefly, a forward mutagenic primer (5′-GGAACTGCAAGTATGACGAGAGC-CCCGTGAC-3′) and a complementary reverse mutagenic primer (5′-GTCACGGGGCTCTCGTCATACTTGCAGTTCC-3′) were synthesized and utilized in PCR experiments according to the manufacturer's instruction. The amplification reaction was treated with DpnI restriction enzyme to eliminate the parental template, and the remaining DNA was used for transformation. Transformed bacterial cultures were grown, and the reporter construct was sequenced to ensure authenticity and purified using the NucleoBond® Xtra Midi Plus (Macherey-Nagel, Germany). The similar procedures were performed for preparation of the reporter plasmid, designated pVP1-EGFP-N1-607T, with a forward mutagenic primer (5′-AGCGAAGC-TAGACACAAATAAAAGATTGTGGCGCCTG-3′) and a complementary reverse mutagenic primer (5′-CAGGCGCCACAATCTTTTATTTGTGTC-TAGCTTCGCT-3′).

2.4. Cotransfection of siRNAs and recombinant plasmids

HEK 293T cells were seeded in 96-well plates at a density of 0.2×10^5 cells/well with 100 μl/well fresh 5% CS-containing DMEM 24 h before transfection. The cells were cotransfected with 160 ng reporter construct and 600 ng each siRNA with 0.8 μl Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. pEGFP-N1-siRNA and Scrambled-siRNA served as positive and negative controls, respectively. The cells were incubated in a 37 °C incubator for various time point observations.

2.5. Fluorescence and flow cytometry analysis

Images for the expression of VP1-EGFP in different siRNAs cotransfected HEK 293T cells were taken with a fluorescence microscope (Olympus IX70, Japan) and a digital camera (Nikon

COOLPIX 4500, Japan) at 12, 24, 36, 48 h after transfection, at a magnification of 10 × 40 with an exposure time of 1/8 s. Flow cytometry analysis at 48 h post-transfection was carried out as follows. After washing with 1× PBS two times, HEK 293T cells were resuspented in 500 μl PBS/well for analysis by FACSCalibur (Becton Dickinson). Cells (10^4) of each well were counted and analyzed with CellQuest software, using mock-transfected HEK 293T cells as control. The values were calculated as the percentage of the cell population that exceeded the fluorescence intensity of the control cells and the mean fluorescence intensity of this population.

2.6. siRNA transfection and viral challenge assays

BHK-21 cells were seeded in six-well plates with 5×10^5 cells/well in 5% serum-containing DMEM 24 h before transfection. The cells were transfected with 15 μg siRNAs using Lipofectamine2000. After 5 h, cells in each well of the six-well plate were infected with 100 $TCID_{50}$ of virus (12 μl viral suspension from the 1000-diluted stock suspension titrated at $TCID_{50} = 10^{6.93}$/ml). After 1 h of adsorption, the inoculum was removed and cells were washed twice with 1× PBS (pH = 5.5). The infection then proceeded in DMEM supplemented with 5% calf serum. At the time points of 12, 24, 36, 48, 60 and 72 h, cells were examined microscopically and light images were taken with fluorescence microscope (Olympus IX70, Japan) at a magnification of 10 × 20 with an exposure time of 1/125 s. Simultaneously, 50 μl sample of supernatants was taken at each indicated time post-infection (p.i.): 12, 24, 36 and 48 h and stored at −80° until the determination of virus titer ($TCID_{50}$). And at 24 h p.i. the cells in each of triplicate wells were harvested for real-time quantitative RT-PCR (RT-qPCR). Viral challenge assay and virus titration were determined three times on BHK-21 cells.

2.7. RNA extraction and RT-qPCR

RNA extraction was performed by using TRIZOL® reagent (Molecular Research Center Inc.) following the manufacturer's instructions. Briefly, 24 h after viral infection, BHK-21 cells were washed with PBS 2 times and then lysed directly by adding 1 ml of TRIZOL reagent. Chloroform (0.2 ml) and 0.5 ml of isopropyl alcohol were used in aqueous phase separation and in precipitation of RNA. After the wash with 75% ethanol, the total RNA was dissolved. Then following a treatment with DNase I (RNase Free) (TaKaRa, Dalian, PR China), the total RNA were re-dissolved for RT-qPCR detection.

The total RNA was reverse transcribed to cDNA with ExScript™RT Reagent Kit (Perfect Real Time) (TaKaRa). The reverse transcription mixture comprised 6.5 μl RNA (500 ng), 2 μl 5× ExScript™ Rtase Buffer, 0.5 μl dNTP Mixture, 0.5 μl Oligo dT, 0.25 μl ExScript™ RTase, and 0.25 μl RNase inhibitor. Real-time PCR mixture consisted of 0.5 μl sense primer (10 μM), 0.5 μl antisense primer (10 μM), 12.5 μl SYBR® Premix ExTaq™ and 9 μl dH_2O (SYBR® Premix Ex Taq™ (Perfect Real Time), TaKaRa Biotechnology Co. Ltd., Dalian, PR China). The primer sequences were as follows: FMDV sense primer, 5′-GGACCCTACACCGGTCC-3′, FMDV antisense primer, 5′-CTCAGTGACGATCAAGTTCTTTGCT-3′; β-actin sense primer, 5′-GCACCACACCTTCTACAATGAG-3′, β-actin antisense primer, 5′-ACAGCCTGGATGGCTACGT-3′. PCR was carried out using an instrument RG-3000A (Gene Company Ltd.) with the annealing temperature at 60°. The data were analyzed using the two standard curve method.

2.8. MTT assay

siRNA transfection and viral challenge were performed in 96-well plates as described in Section 2.6 except that the amounts of seeded BHK-21 cells, siRNAs and viruses in each well were 1/25 of those in six-well plates. At 12, 24, 36 and 48 h p.i., cell viability was separately assessed by adding 20 μl/well of MTT (methyl thiazolyl tetrazolium) (5 mg/ml pH = 7.4; Huashun Biotech Co. Ltd., Shanghai, PR China). After 4 h incubation at 37°, supernatant of each well was removed and 150 μl DMSO (dimethyl sulphoxide) was added in each well followed by 10 min of shaking. Light absorbance of each well was measured at 490 nm. The experiment was repeated three times in duplicates.

2.9. Cloning and sequencing of VP1, VP4, 3B and 3C

FMDV viral RNA was extracted from the BHK-21 cells infected with FMDV(NY00) using Body fluid viral DNA/viral RNA mini-prep Kit (V-gene Biotechnology Ltd., PR China). Then the viral RNA was reverse transcribed to cDNA using ExScript™RT Reagent Kit (Perfect Real Time) (TaKaRa) according to the manufacturer's manual. The PCR primer pairs were as follows: VP1 sense primer 5′-CCGGAATTCATGACCACCTCCACAGG-3′, antisense primer 5′-CGCGGATCCTCCAAAAGCTGTTTCACAG-3′; VP4 sense primer 5′-CCGGAATTCATGAACACTGGCAGCATTATCA-3′, antisense primer 5′-CGCGGATCCTCGGCGAGAAGAGCGCC-3′; 3B sense primer 5′-CCGGAATTCATGGGACCCTACACCGG-3′, antisense primer 5′-CGCGGATCCTCCTCAGTGACGATCAAGTTC-3′; 3C sense primer 5′-CCGGAATTCATGAGTGGTGCTCCCCCG-3′, antisense primer 5′-CGCGGATCCTCCTCGTGGTGTGGTTCG-3′. The purified PCR products of VP1, VP4, 3B and 3C were cloned into pEGFP-N1 vector and recombinant clones were sequenced by big dye terminator cycle-sequencing using the amplification primer, and analyzed on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol. The sequence primers were: sense primer 5′-TGGGAGGTCTATATAAGCAGAG-3′, antisense primer 5′-CGTCGCCGTCCAGCTCGACCAG-3′. The gene sequences were reconfirmed using Chromas1.62 (Technelysium Pty Ltd., Australia) and the sequences were aligned with CLUSTAL X (1.83).

2.10. Statistical analysis

Statistical comparisons between experimental groups and control groups were performed using one way ANOVA and Dunnett's method (multiple comparisons versus control group), with P less than 0.05 regarded as a statistically significant difference. Data are presented as mean ± SD.

3. Results

3.1. In vitro enzymatic generation of 21-nt small interfering RNA (siRNA)

To quickly and efficiently synthesize siRNAs candidates targeting sequences of VP1 region in FMDV (NY00) genome, DNA oligonucleotide templates for in vitro synthesis with T7 RNA polymerase were designed. We obtained 14 sequences for in vitro siRNA synthesis, whose position of the first nucleotide in VP1 were designated their corresponding siRNAs name suffixes (VP1-siRNA55, VP1-siRNA63, VP1-siRNA80, VP1-siRNA113, VP1-siRNA192, VP1-siRNA401, VP1-siRNA423, VP1-siRNA465, VP1-siRNA477, VP1-siRNA516, VP1-siRNA517, VP1-siRNA519, VP1-siRNA540 and VP1-siRNA616). For each of the sequence candidates, two complementary RNA strands synthesized from DNA templates were annealed to form siRNA. The produced double-stranded 21-nt siRNAs with 19 bases of homology to FMDV and 3′ overhang UU were observed and found intact (Fig. 1).

3.2. Expression of VP1-EGFP and 3C-EGFP in cultured HEK 293T cells

To monitor fusion protein expression as an indication of interference by siRNAs candidates, VP1 gene were cloned into pEGFP-

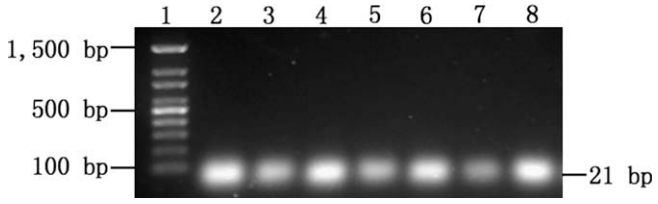

Fig. 1. Agarose gel analysis of siRNA molecules generated using the T7 RNA polymerase. Each siRNA was separated on a 1% agarose gel/1× TAE and visualized by staining the gel with 0.5 μg/ml ethidium bromide. Lane designations: lane 1, 100 bp DNA ladder (GenScript Corp.); lane 2, pEGFP-N1-siRNA; lane 3, scrambled-siRNA; lane 4, VP1-siRNA517; lane 5, VP1-siRNA113; lane 6, VP1-siRNA616; lane 7, VP1-siRNA519; lane 8, VP1-siRNA401. *Note:* siRNA migrates more slowly than double-stranded DNA. Only those five efficacious siRNAs out of siRNAs candidates targeting the VP1 of FMDV are shown.

N1 with a C-terminal EGFP tag (Fig. 2A), and then the constructed plasmid pVP1-EGFP-N1 was transfected into HEK 293T cells using Lipofectamine2000 according to the instructions (Invitrogen, Carlsbad, CA, USA). Strong fluorescence was observed by fluorescence microscopy at 48 h after transfection (Fig. 2B). To confirm sequence-specific silencing by siRNAs, we designed p3C-EGFP-N1, in which 3C sequence was cloned upstream of the EGFP gene without the target sequences for VP1-specific siRNAs. The same high level of fluorescence as that of pVP1-EGFP-N1 was observed in p3C-EGFP-N1 transfected cells (images not shown). Thus, these two transient expression systems transfected with pVP1-EGFP-N1 and p3C-EGFP-N1 were suitable to test the efficiency of inhibition and the sequence specificity of inhibition by siRNAs, respectively.

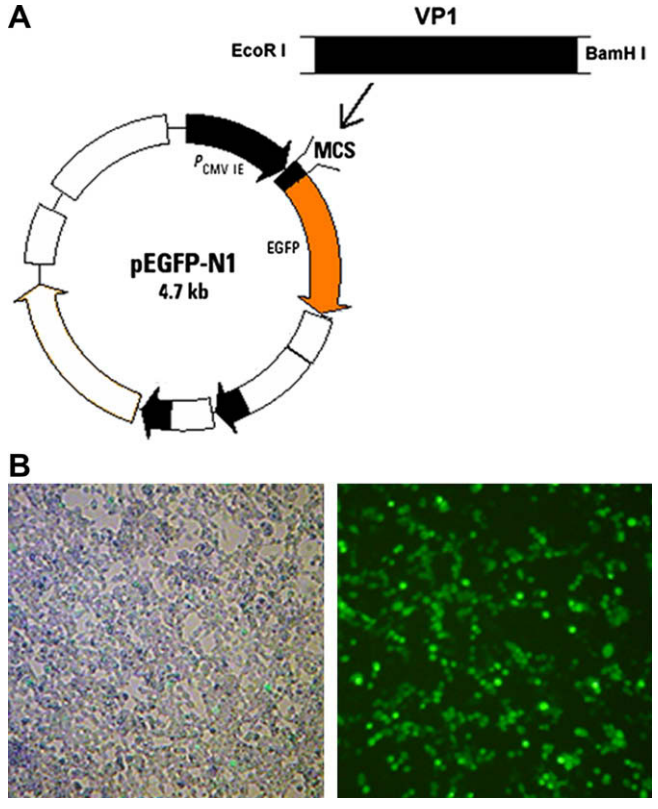

Fig. 2. Expression of pVP1-EGFP-N1 in HEK 293T cell line. (A) The pVP1-EGFP-N1 reporter constructs. The complete VP1 sequence of FMDV genome was inserted upstream of the EGFP gene. (B) pVP1-EGFP-N1 expressing VP1-EGFP in HEK 293T cells. Bright-field image (left) and the corresponding fluorescent-field image (right) were taken with fluorescence microscope (Olympus IX70, Japan) at 48 h after transient transfection.

3.3. Sequence-specific inhibition of VP1-EGFP mediated by synthetic siRNAs

To test the ability of each siRNA to inhibit VP1-EGFP expression, we cotransfected HEK 293T cells with positive control pEGFP-N1-siRNA, negative control Scrambled-siRNA, or the siRNAs candidates targeting VP1 along with pVP1-EGFP-N1 expression plasmid. This screening test resulted in the identification of five siRNAs effective at inhibiting VP1-EGFP expression as assessed by fluorescence microscopic observation and flow cytometric analysis (Fig. 3A–C). VP1-siRNA517, VP1-siRNA113, VP1-siRNA616, VP1-siRNA519 and VP1-siRNA401 showed significant reduction of green fluorescence and mean fluorescence intensity. In the same experiments for the other nine siRNAs: VP1-siRNA55, VP1-siRNA63, VP1-siRNA80, VP1-siRNA192, VP1-siRNA423, VP1-siRNA465, VP1-siRNA477, VP1-siRNA516 and VP1-siRNA540, those results showed little inhibition (below 60%) or no inhibition (images and data not shown). By replacing the pVP1-EGFP-N1 plasmid with p3C-EGFP-N1 which contained no target sequences for the five siRNAs targeting VP1, the similar assay was re-conducted to test whether the inhibition by these siRNAs was sequence specific. When the siRNAs did not match the reporter sequences, the reductions of green fluorescence and mean fluorescence intensity were not induced (Fig. 3D). The results confirmed that the observed inhibition of VP1-EGFP expression occurred through a sequence-specific manner. Since VP1-siRNA517, VP1-siRNA113, VP1-siRNA616, VP1-siRNA519 and VP1-siRNA401 induced high level of inhibition they were used for further viral challenge analysis.

3.4. siRNAs exert transient silencing of FMDV

3.4.1. Inhibition of viral RNA replication

To examine whether siRNAs synthesized in vitro could inhibit FMDV replication in cell culture, BHK-21 cells were inoculated with 100 $TCID_{50}$ FMDV O (NY00) following the transfection with each siRNA. At 24 h p.i., the amounts of BHK-21 cells from each well of triplicates were harvested and RNA was extracted. This served as a template for real-time RT-qPCR. As shown in Fig. 4 A, the mean relative amounts of viral RNA in BHK-21 transfected with pEGFP-N1-siRNA, Scrambled-siRNA, VP1-siRNA517, VP1-siRNA113, VP1-siRNA616, VP1-siRNA519 and VP1-siRNA401 were 95.0%, 93.0%, 2.55%, 4.76%, 86.0%, 4.93% and 23.48%, respectively, compared with the levels of viral RNA in control–experiment cells, which were viral challenged without treatment of siRNAs. Obviously, the marked effective inhibition found by far was that of cells transfected with VP1-siRNA517, VP1-siRNA113, VP1-siRNA519 and VP1-siRNA401. Additionally, to test whether a combinatory strategy could exert enhanced inhibitory effect, we mixed the three most efficacious siRNAs (VP1-siRNA517, VP1-siRNA113 and VP1-siRNA519) together with equal amount, and performed the same procedures. And the mean relative amount of viral RNA in BHK-21 transfected with the mixtures was 3.5%, compared with the control.

3.4.2. Transient reduction of viral titer by efficacious siRNAs

In addition to the examination of FMDV RNA replication, we also tested the silencing effect of FMDV virus by siRNAs on the yield of progeny virus. At 12, 24, 36 and 48 h p.i., the supernatants of BHK-21 cells were titrated out on a 96-well plate containing BHK-21 cells. $TCID_{50}$, made from the cytopathic effect (CPE) in BHK-21 by different dilutions of the harvested virus, was used to calculate the amount of virus (Reed and Muench, 1938). As shown in Fig. 4B, the virus yield from mock-transfected cells or transfected cells with pEGFP-N1-siRNA or Scrambled-siRNA was similar, and reached maximum about at 36 h p.i.. Consistent with results

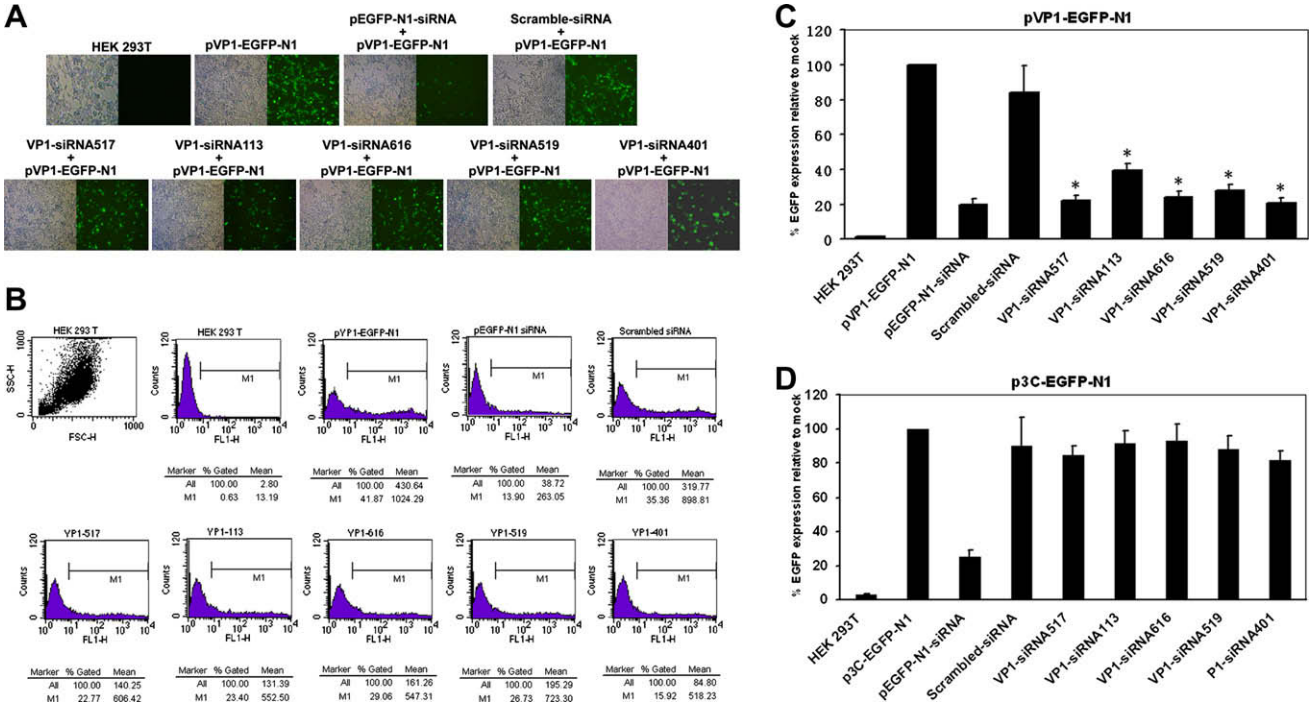

Fig. 3. Screening of sequence-specific siRNAs inhibiting the expression of VP1-EGFP in HEK 293T cells. (A) Shown here is the bright-field image (left) and the relative fluorescent-field image (right) at 48 h after a cotransfection assay of all siRNAs with pVP1-EGFP-N1. (B) Flow cytometry analysis of VP1-EGFP expression at 48 h after cotransfection. The percentage of the cell population that exceeded the fluorescence intensity of the control cells (HEK 293T) and the mean fluorescence intensity of this population were calculated. (C) Relative GFP expression was measured with the corresponding data from flow cytometry. VP1-EGFP production in the absence of siRNA inhibitor was set at 100% (pVP1-EGFP-N1) for the negative control. (D) Relative GFP expression was measured after a cotransfection of all siRNAs with p3C-EGFP-N1 in HEK 293T cells. The mean values and standard deviations obtained from three independent cotransfections are shown. $^{*}P < 0.05$ vs. scrambled-siRNA group.

obtained from the viral RNA quantitation, BHK-21 cells that were transfected with VP1-siRNA517, VP1-siRNA113, VP1-siRNA519 or the mixtures displayed a markedly lower level of progeny virus than cells transfected with mock-infected cells or the above two control siRNAs at 12, 24 and 36 h p.i.. Slight low level of progeny virus was observed in cells transfected with VP1-siRNA401. On the other hand, VP1-siRNA616 only showed almost no inhibition of the yield of progeny virus.

3.4.3. Short delays of CPE by efficacious siRNAs

Importantly, observations of the CPE, which displayed complete detachment, rounding up and disintegration from the BHK-21 cells monolayer, were as follows: At 24 h p.i., slight CPE emerged from mock-transfected cells, transfected cells with pEGFP-N1-siRNA or scrambled-siRNA or VP1-siRNA616, and at 36 h p.i., extensive CPE occupied all the above samples. However, the emergence of slight CPE from cells transfected with VP1-siRNA517, VP1-siRNA113, VP1-siRNA519 or the mixtures and moderate CPE with VP1-siRNA401 at 36 h p.i. showed a delay of approximate 12 and 6 h, respectively (Fig. 5A). By 48 h p.i., all samples including mock-transfected cells or transfected cells with siRNAs were showing extensive CPE. Viability percentage detected with MTT assay showed that obvious elevation of BHK-21 viability could be reached in groups of VP1-siRNA517, VP1-siRNA113, VP1-siRNA519 and the mixtures, particularly at 36 h p.i. (Fig. 5B).

These above results showed that, among the five siRNA candidates validated by reporter gene assay, VP1-siRNA517, VP1-siRNA113, VP1-siRNA519 or the mixtures had marked inhibitory effect on the replication of FMDV, although the delay for duration of inhibition was transient. But there was no significant difference between the mixtures and each of the components (VP1-siRNA517, VP1-siRNA113 or VP1-siRNA519) in terms of the inhibitory efficacy (Table 1).

3.5. Changes of nucleotides in FMDV genome could cause RNAi resistance

3.5.1. Probing the sequences of multiple genes from FMDV

We then explored retrospectively to see whether or not the variations existing in the VP1 gene could influence the silencing effect by each siRNA candidate. In addition, we also randomly compared the nucleotide similarity of other genes (VP4, 3B and 3C) between variants and wildtype. VP4 gene encodes the structural protein VP4, which is internal in 60 asymmetrical protomers and has an N-terminal myristic acid; 3B gene encodes 3B (VPg), with the number of which the level of viral infectivity correlates; 3C gene product is a thiol-protease responsible for most of the cleavage events undergone by the viral polyprotein and also for the proteolytic process of histone H3. The original supernatants, which were collected from FMDV-transfected BHK-21 cells and used for virus infection in our experiment, were subjected to rapid nucleotide sequencing. In brief, reverse transcription of FMDV genomic RNA and PCR amplification, followed by molecular cloning from more than 5 clones and sequencing of each individual clone, presented the nucleotide sequences of VP1, VP4, 3B and 3C. As shown in Fig. 6, the variation point numbers within genes for VP1, VP4, 3B and 3C were 11 nts (nucleotides)/639 nts, 8 nts/207 nts, 1 nts/213 nts and 31 nts/639 nts, respectively. Intriguingly, all the regions in VP1 targeted by candidate siRNAs did not contain variations, despite that only a nucleotide substitution locating at 410 nt in VP1 gene was being within VP1-siRNA401 target region. Furthermore, another variation point locating at 607 nt was found near the 5′-end of the VP1-siRNA616 target sequence.

3.5.2. A single nucleotide substitution in VP1-siRNA401 target region could cause RNAi resistance

To explore whether the single nucleotide substitutions within or near the siRNA target region lead to the resistance of FMDV from

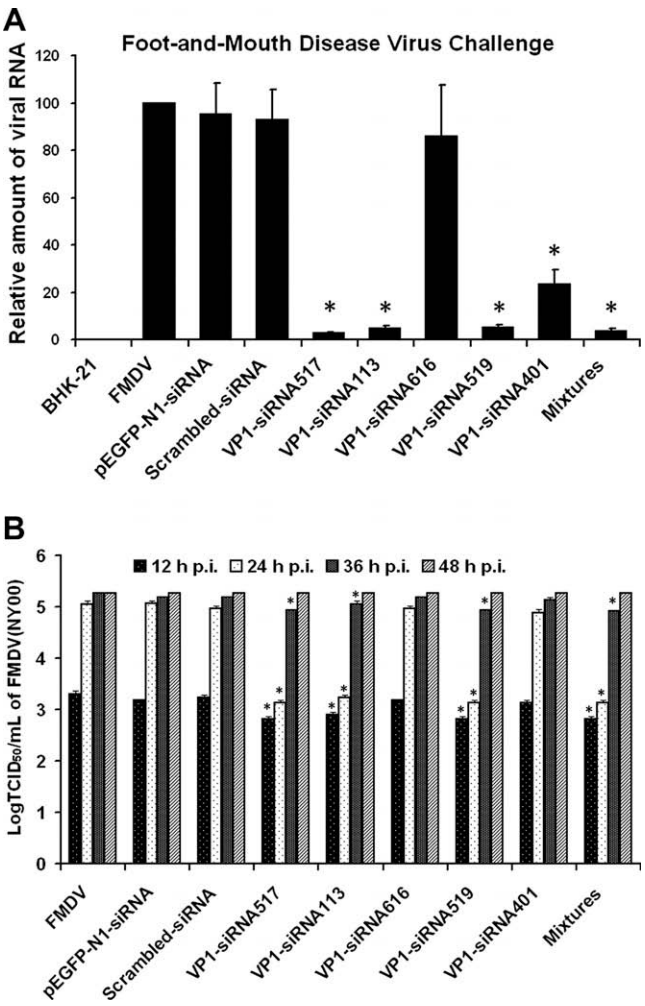

Fig. 4. Inhibition of FMDV replication. (A) Relative amounts of viral RNA in siRNA-transfected BHK-21 cells. BHK-21 cells in six-well plates were transfected with each of the siRNAs or the mixtures for 5 h and then inoculated with 100 $TCID_{50}$ FMDV. At 24 h p.i., half of each of the samples was used for RNA extraction to determine the relative amounts of viral RNA by real-time RT-qPCR with normalization over β-actin. The relative amount of viral RNA in the viral challenged cells without siRNA was set as 100. (B) Decrease of virus yield in BHK-21 cells at the designated times p.i. Culture supernatants were collected at the time point of 12, 24, 36 and 48 h p.i., and virus yields were measured by $TCID_{50}$. The titers on *y*-axis are shown by Log $TCID_{50}$/ml. Error bars represent the standard deviation from three independent experiments. $^{*}P < 0.05$ vs. scrambled-siRNA group.

siRNA, we made reporter gene constructs variants designated pVP1-EGFP-N1-410A and pVP1-EGFP-N1-607T. The former was introduced G → A substitution at the position of 410 of the pVP1-EGFP-N1 (temporarily designated pVP1-EGFP-N1-410G), and the latter was introduced C → T substitution at 607 of the pVP1-EGFP-N1 (temporarily designated pVP1-EGFP-N1-607C). These constructs were cotransfected with their corresponding siRNAs (VP1-siRNA401 or VP1-siRNA616) into HEK 293T cells and reporter gene expression level were measure by flow cytometry analysis after 48 h. As shown in Fig. 7A, pVP1-EGFP-N1-410A nearly obtained about 3.7-fold increase level of resistance from VP1-siRNA401 compared with pVP1-EGFP-N1-410G, indicating that the single nucleotide substitution G → A (position 410 nt in VP1) within VP1-siRNA401 target region markedly reduces the interfering efficiency of VP1-siRNA401. Nevertheless, the nucleotide substitution C → T at position 607 nt in VP1, which located outside of the VP1-siRNA616 target region, did not show significant differences in reporter gene expression level between pVP1-EGFP-N1-607C and pVP1-EGFP-N1-607T (Fig. 7B).

3.6. Second round infection showed no enhanced resistance to efficacious siRNAs

Notably, we repeated the assay of viral RNA replication, viral titer and cytopathic effects using the virus supernatants from the first round of inhibition to initiate the infection. The results showed that the efficacy of VP1-siRNA517, VP1-siRNA519 and VP1-siRNA113 in the second round exhibited almost the similar level as that in the first round, separately (data not shown). This means the delayed viruses do not show significantly increased resistance to each efficacious siRNA.

4. Discussion

We tested siRNAs targeting VP1 of FMDV, five out of 14 siRNAs steadily resulted in effective and specific silencing effects in the reporter assay in HEK 293T cells. Furthermore, among the five siRNAs candidates, three siRNAs were confirmed efficacious to transiently inhibit the replication of FMDV in BHK-21 cells by determining the relative viral amount within infected cells and the virus titers in supernatants as well as the observation of the emergence of CPE.

We first used an inexpensive method of T7 RNA polymerase-directed in vitro transcription to obtain siRNAs targeting FMDV, which was first presented and demonstrated silencing of both exogenous and endogenous genes (Donze and Picard, 2002). Different from previous RNAi inhibiting FMDV studies (Chen et al., 2004, 2006; de los Santos et al., 2005; Kahana et al., 2004), in which anti-FMDV RNAi molecules were produced with chemical synthesis or shRNA-vector expression, exploration of this method as an anti-FMDV RNAi strategy was due to the following considerations. Firstly, the high genetic and phenotypic variability of FMDV inevitably poses the need for screening a relatively large amount of siRNAs for those efficaciously inhibiting the amplification of FMDV in a specific FMD outbreak. Secondly, the chemical synthesis of siRNAs involves high cost, and the large quantities of shRNAs cloned into plasmid or viral vectors are time-consuming, both of which cannot guarantee the sufficient inhibitory effect. Therefore, it is more feasible to begin the search for highly effective siRNAs in transient RNAi experiments with the less expensive and faster method of T7 RNA polymerase-directed in vitro transcription.

Although searching for highly conserved sequences would be the best strategy against virus escape just like Kahana et al. have done, the reason why we only chose VP1 region is that VP1 gene is the most important region for FMDV immunogenicity. We searched the entire sequence of VP1 gene, according to the sequence requirement that the presence of a "5′-GN17C-3′" sequence in the target RNA is needed, and obtained all possible template sequences. Consequently, we synthesized 14 candidate siRNAs for efficacy test. Among VP1-siRNA517, VP1-siRNA113, VP1-siRNA616, VP1-siRNA519 and VP1-siRNA401, which demonstrated significant reduction of reporter gene, VP1-siRNA517, VP1-siRNA113, VP1-siRNA519 and their mixtures resulted in efficient inhibition of FMDV replication in BHK-21 cells, and the delayed duration of inhibition reached about 12 h in terms of the emergence of the maximum virus yield and the complete CPE. Although the duration of inhibition was not as 2–3 days long as other vector-based RNAi studies (Chen et al., 2004, 2006), the efficacious siRNA target sequences were identified.

In addition to each efficacious siRNA, we treated BHK-21 cells with the siRNA mixtures, and the results demonstrated that the inhibitory effects of the mixtures on FMDV RNA replication, viral titer and CPE were approximately the same as that of each component siRNA. The reasons that the mixtures did not exert significant increased inhibitory effect, such as that of three highly conserved siRNAs targeted to 3B and 3D region in FMDV genome (Kahana

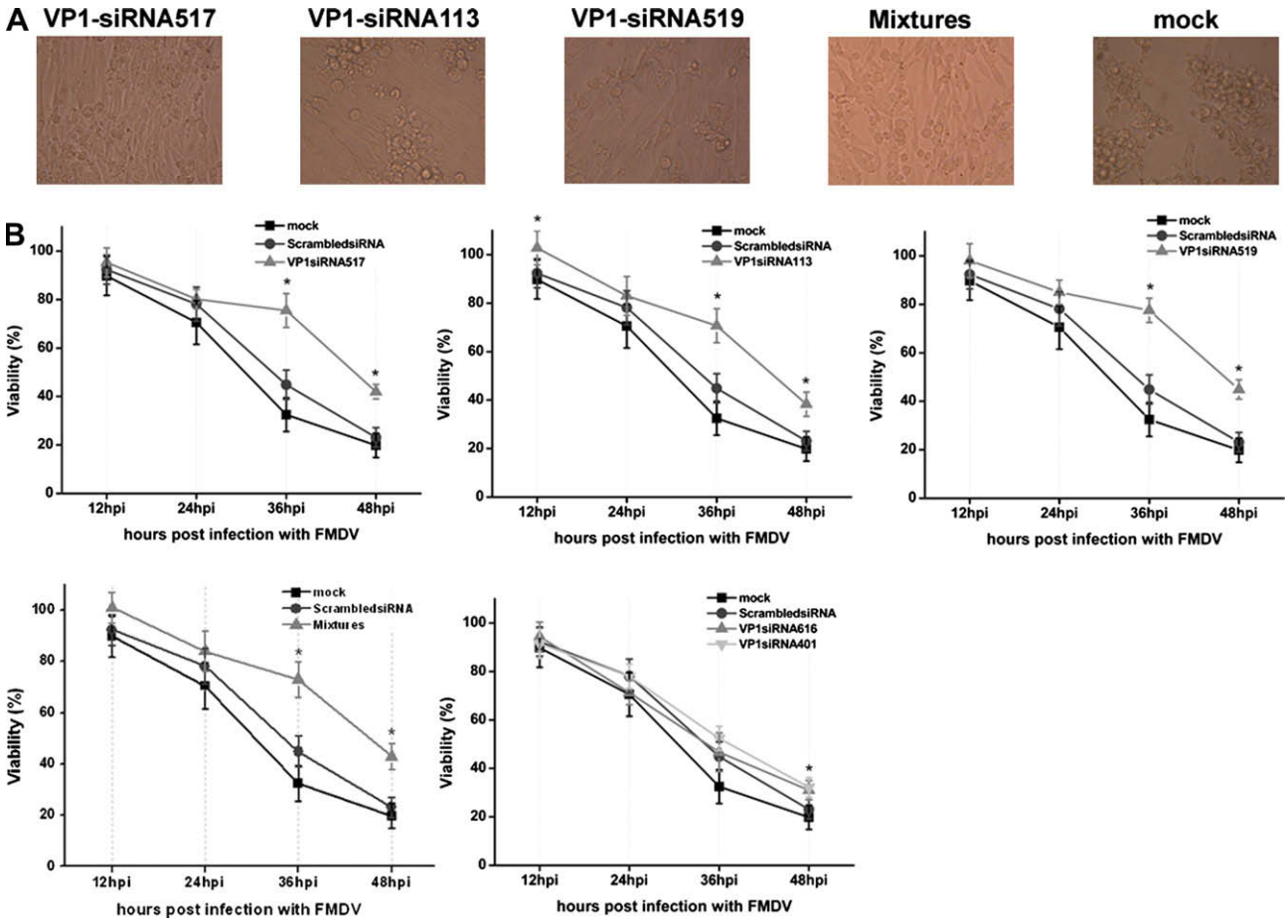

Fig. 5. Short period protection of BHK-21 cells from CPE by efficacious siRNAs. (A) pVP1-siRNA517, pVP1-siRNA113, pVP1-siRNA519, mixtures and mock, respectively, represent BHK-21 cells transfected with the corresponding siRNAs at 36 h post-infection with FMDV. All images were obtained using an Olympus IX70 microscope at a magnification of 10 × 20. (B) At 12, 24, 36, 48 h p.i., BHK-21 cells viability was evaluated by MTT assay. Viability percentage values shown are the mean ± standard deviation of three separate experiments performed in duplicate. $^{*}P < 0.05$ vs. scrambled-siRNA group.

Table 1
siRNAs molecules targeting VP1 of FMDV and their template oligonucleotides.

siRNAs	siRNA sequences	Template oligonucleotide structures[b]
VP1-siRNA517	5′-GUGACUGAACUGCUUUACCUU-3′, 3′-UUCACUGACUUGACGAAAUGG-5′	5′-AAGGTAAAGCAGTTCAGTCACTATAGTGAGTCGTATTAGGATCC-3′, 5′-AAGTGACTGAACTGCTTTACCTATAGTGAGTCGTATTAGGATCC-3′
VP1-siRNA113	5′-GAUUUGUGAAAGUAACACCUU-3′, 3′-UUCUAAACACUUUCAUUGUGG-5′	5′-AAGGTGTTACTTTCACAAATCTATAGTGAGTCGTATTAGGATCC-3′, 5′-AAGATTTGTGAAAGTAACACCTATAGTGAGTCGTATTAGGATCC-3′
VP1-siRNA616	5′-GUGGCGCCUGUGAAACAGCUU-3′, 3′-UUCACCGCGGACACUUUGUCG-5′	5′-AAGCTGTTTCACAGGCGCCACTATAGTGAGTCGTATTAGGATCC-3′, 5′-AAGTGGCGCCTGTGAAACAGCTATAGTGAGTCGTATTAGGATCC-3′
VP1-siRNA519	5′-GACUGAACUGCUUUACCGCUU-3′, 3′-UUCUGACUUGACGAAAUGGCG-5′	5′-AAGCGGTAAAGCAGTTCAGTCTATAGTGAGTCGTATTAGGATCC-3′, 5′-AAGACTGAACTGCTTTACCGCTATAGTGAGTCGTATTAGGATCC-3′
VP1-siRNA401	5′-GCAAGUAUGGCGAGAGCCCUU-3′, 3′-UUCGUUCAUACCGCUCUCGGG-5′	5′-AAGGGCTCTCGCCATACTTGCTATAGTGAGTCGTATTAGGATCC-3′, 5′-AAGCAAGTATGGCGAGAGCCCTATAGTGAGTCGTATTAGGATCC-3′
pEGFP-N1-siRNA	5′-GCUGACCCUGAAGUUCAUCUU-3′, 3′-UUCGACUGGGACUUCAAGUAG-5′	5′-AAGATGAACTTCAGGGTCAGCTATAGTGAGTCGTATTAGGATCC-3′, 5′-AAGCTGACCCTGAAGTTCATCTATAGTGAGTCGTATTAGGATCC-3′
Scrambled-siRNA	5′-GGUUCAUCGGCACUUCAACUU-3′, 3′-UUCCAAGUAGCCGUGAAGUUG-5′	5′-AAGTTGAAGTGCCGATGAACCTATAGTGAGTCGTATTAGGATCC-3′, 5′-AAGGTTCATCGGCACTTCAACTATAGTGAGTCGTATTAGGATCC-3′
T7 RNA Pol oligo[a]		5′-GGATCCTAATACGACTCACTATAG-3′

[a] T7 RNA Pol oligo: the T7 RNA polymerase promoter sequence and six extra nucleotides upstream of the minimal promoter sequence.
[b] The underlined regions of each template are the corresponding T7 promoter sequences.

et al., 2004), are as follows. Firstly, our three efficacious siRNAs were randomly screened without consideration of sequence conservation. Secondly, each of the three siRNAs constituting the mixtures might possess the equal efficacy to inhibit replication of FMDV.

The reason why no enhanced resistance to efficacious siRNAs was found in the second round of infection, in our opinion, might be as follows. First, the most important factor for virus escape from efficacious siRNAs in our experiments might be the quasispecies of FMDV, but not those new emerging resistant variants. As our results showed, the inhibitory effect of each efficacious siRNA did not persist longer than 48 h post-infection, which could not allow the occurrence of large number of mutants despite high mutation rates of FMDV. Second, the restricted transfection efficiency of siRNAs could not guarantee complete occupation of siRNAs in BHK-21 cells infected with FMDV. Moreover, the relatively quick speed of

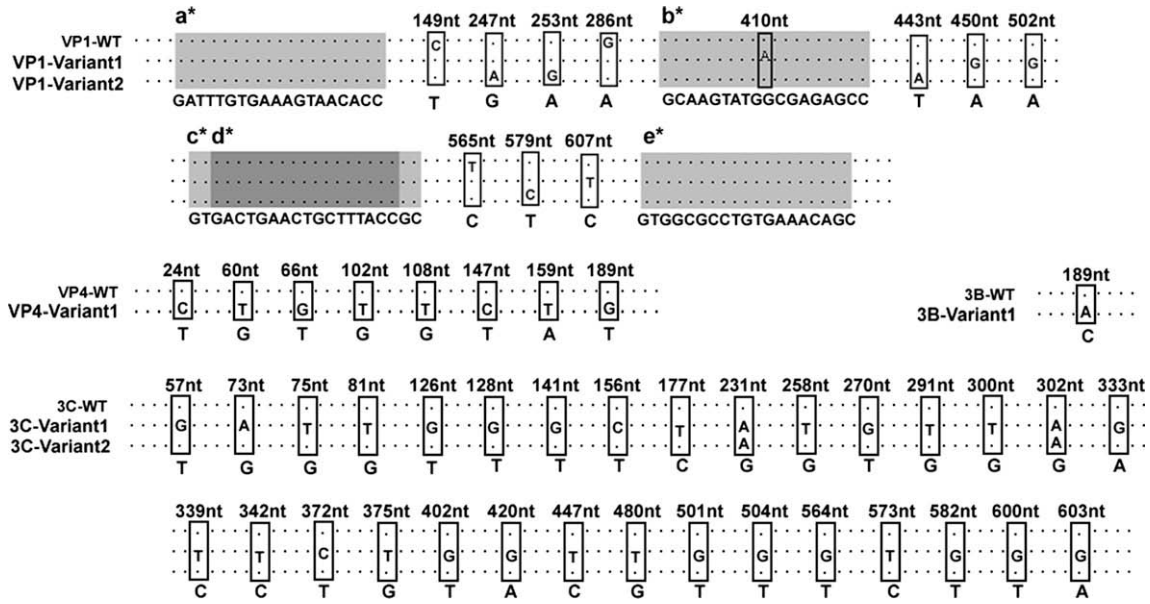

Fig. 6. Alignment of FMDV (isolate O/NY00) wildtype (WT) and the variants nucleotide sequences. Alignments in regions of VP1, VP4, 3B and 3C were performed and adjusted manually. The parts with variations are boxed in small rectangle (□). SiRNAs targeted sequences in VP1 region were indicated in gray. Indicated target regions: a*, VP1-siRNA113; b*, VP1-siRNA401; c*, VP1-siRNA517; d*, VP1-siRNA519; e*, VP1-siRNA616.

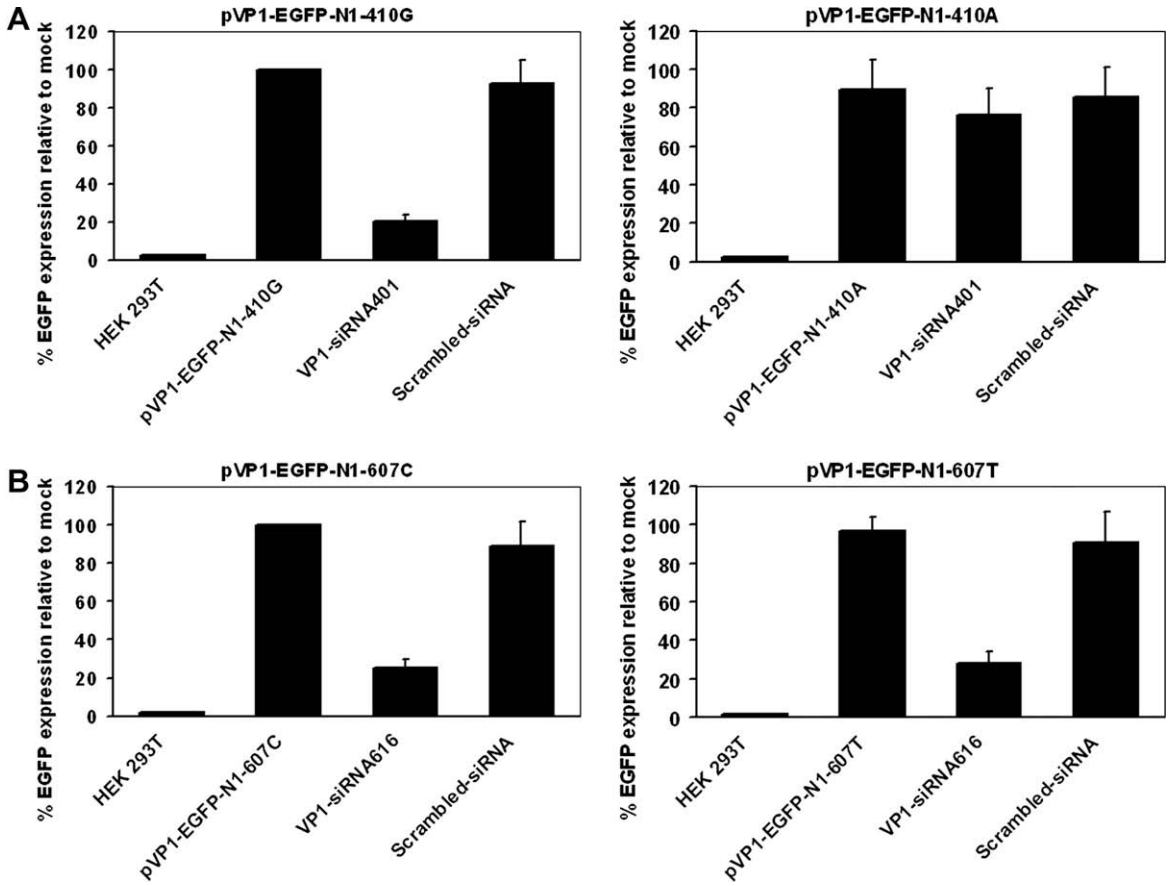

Fig. 7. Influences of single nucleotide substitution within or near the siRNA target region on RNAi resistance. (A) HEK 293T cells were cotransfected with pVP1-EGFP-N1-410G or pVP1-EGFP-N1-410A with VP1-siRNA401. (B) HEK 293T cells were cotransfected with pVP1-EGFP-N1-607C or pVP1-EGFP-N1-607T with VP1-siRNA616. Relative GFP expression was measured by Flow cytometry analysis at 48 h after cotransfection. The mean values and standard deviations obtained from three independent cotransfections are shown.

degradation of siRNAs could not persist long enough at an efficacious concentration, raising the possibility of virus escape.

Furthermore, detailed analysis of mutations of FMDV should be emphasized in order to raise specific measures to facilitate RNAi to surmount the viral escape, which ultimately reduces the efficacy for long-term inhibition. In this study, quasispecies dynamics or possibly high mutation rates of FMDV RNA might facilitate the escape of FMDV from siRNA, which is characterized by a high degree of sequence specificity. As we observed, different variants existed in the supernatant of viral infected BHK-21 cells. Particularly, as we tested, a single nucleotide substitution G → A found within the VP1-siRNA401 target region can be the main factor causing the marked decrease of anti-FMDV efficacy. Moreover, although the single nucleotide substitution C → T near the target region of VP1-siRNA616 did not result in resistance of the target sequence to VP1-siRNA616, we cannot absolutely deny the possibility that the existence of variations within VP1-siRNA616 target sequence could be identified by molecular cloning and sequencing of more clones. It was also possible that the C → T substitution could affect the RNA structure, affecting the accessibility of RISC to FMDV RNA and the efficiency of interference. A study of HIV-1 escape mutant viruses (Westerhout et al., 2005) revealed that not only the nucleotide substitutions or deletions in the siRNA target sequence, but also the mutations outside the target region stabilizing a repressive structure in viral RNA genome could mediate escape from RNAi. Variations within VP1, identified by us, were not located in the target sequence for VP1-siRNA517, VP1-siRNA113 and VP1-siRNA519. Additionally, the restricted transfection efficiency and the relatively quick speed of degradation of siRNAs could not guarantee that siRNAs entered all the FMDV-infected BHK-21 cells or persisted long enough at an efficacious concentration, raising the possibility of virus escape.

Aside from these factors above, the reasons why the fluctuation of inhibition efficiency existed between the reporter assay and viral challenge assay in this study, particularly the inefficacy of VP1-siRNA616 and VP1-siRNA401 in FMDV challenged BHK-21 cells, are presumably as follows. Differences in the VP1 spatial structures between the reporter plasmid pVP1-EGFP-N1 and FMDV genomic RNA might affect the accessibility of RNA-induced silencing complex (RISC) to combine with VP1.

The practical use of siRNA (RNAi) for virus infection, such as the RNAi therapy for FMDV infection and HIV infection (Bennasser et al., 2007; Brass et al., 2008), will depend on overcoming a couple of crucial obstacles, including the establishment of long-term expression of siRNA without off-target effects and the methods to counteract mutant escape viruses, and so on. Fortunately, many strategies, such as the non-viral or viral vector-based expression of shRNA in vivo, and the selection of conserved sequences and the combinatorial use of several viral and/or feasible cellular targets, are being exploited to overcome the above problems (Brass et al., 2008; Giering et al., 2008). Promisingly, in humans, some preclinical studies and clinical trials have exhibited satisfying results in terms of the tolerability and inhibitory activity in vivo (DeVincenzo et al., 2008; Meyers et al., 2007). So, the practical use of siRNAs (RNAi) will have a promising future. Noticeably, siRNAs (RNAi) should be most probably used in the conditions where vaccines and drugs are unavailable or inadequate (Manjunath et al., 2006).

In conclusion, our results showed that the replication of FMDV could be transiently inhibited by VP1-siRNA517, VP1-siRNA113 and VP1-siRNA519, which were obtained with T7 RNA polymerase-directed in vitro transcription. Also, FMDV could rapidly escape from the transient inhibition of RNAi due to single nucleotide substitution within target sequences of the multiple variants and quasispecies of FMDV, from which the necessity of further strategies should arise.

Acknowledgments

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