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ORIGINAL ARTICLE

Construction of a multiple targeting RNAi plasmid that inhibits target gene expression and FMDV replication in BHK-21 cells and suckling mice

Wei Cong · Shaoqiang Cui · Jiulian Chen · Xiaoping Zuo · Yonggan Lu · Weiyao Yan · Zhaoxin Zheng

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Abstract Foot-and-mouth disease (FMD) is a highly contagious disease that afflicts cloven-hoofed animals. The etiological agent of FMD is foot-and-mouth disease virus (FMDV). The VP1 gene of FMDV is essential during the life cycle of the virus and plays a key role in the attachment of the virus to susceptible cells. We constructed a plasmid, pCWN11, that expresses siRNAs multiple-targeting the VP1 genes of FMDV. We evaluated the gene silencing efficiency of the plasmid using an enhanced green fluorescent protein (EGFP) reporter system in BHK-21 cells. The antiviral potential of the plasmid in BHK-21 cells and suckling mice were investigated. The results indicate that cotransfection of pCWN11 with any one of three serotypes VP1-EGFP plasmids resulted in a reduction in the EGFP signal relative to the control. Moreover, the antiviral potential induced by pCWN11 was evident during challenge with one FMDV isolate of either serotype O (HKN/2002) or serotype Asia I (YNBS/58), and the inhibition extended to almost 40 h. Furthermore, subcutaneous injection of pCWN11 in the neck made suckling mice significantly less susceptible to FMDV serotype O and Asia I.

Keywords FMDV · VP1 gene · RNA interference (RNAi) · siRNA · Multiple targeting plasmid

W. Cong · S. Cui · W. Yan · Z. Zheng (✉)
State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Science, Fudan University, 220 Handan Road, Shanghai 200433, People's Republic of China
e-mail: zxzheng@fudan.edu.cn

W. Yan
e-mail: wyyan@fudan.edu.cn

J. Chen · X. Zuo · Y. Lu
Bio-pharmacy, Jinyu Group Co. LTD, Hohhot, Inner Mongolia 010020, People's Republic of China

Introduction

Foot-and-mouth disease virus (FMDV) is a picornavirus that causes of a highly contagious disease of cloven-hoofed animals. For centuries, outbreaks of FMDV have caused heavy economic losses worldwide. FMDV shows a high genetic and antigenic variability, which is reflected in the seven serotypes and the numerous variants described to date (Bachrach 1968). The FMDV genome is composed of a positive-sense single-stranded RNA molecule of about 8500 nucleotides, which contains a unique open reading frame. There are seven distinguishable serological types, namely, O, A, C, Asia I, SAT1, SAT2, and SAT3, and more than 65 subtypes.

RNA silencing or post-transcriptional gene silencing (PTGS) is a process of degradation of cognate mRNA in response to the introduction of a double-strand RNA (dsRNA). It is commonly accepted that RNA silencing is the major antiviral defense system in plants and invertebrates. RNA interference (RNAi) is an innate cellular process activated by a double-stranded RNA duplex in cells from *Caenorhabditis elegans* to mammals (Fire et al. 1998). Specific inhibition of cellular mRNA by RNAi can be triggered in mammalian cells by the introduction of synthetic 21–23-nucleotide double-stranded small interfering RNA (siRNA) (Elbashir et al. 2001a; Paul et al. 2002) or, alternatively, by the transcription of siRNA from a DNA construct driven by the RNA polymerase cassette (Brummelkamp et al. 2002). RNAi is initiated by the degradation of single-stranded RNA of identical sequence. Therefore, the RNAi approach can be used to silence gene expression by directly targeting its specific sequence of mRNA. The RNAi approach has been reported as an ideal tool to inhibit infectious virus replication in host cells because siRNA can target and silence important genes of the virus. Because of the high rapidity and specificity of the RNAi effect, this method may complement and improve the traditional tools available to control important animal pathogens.

As an antiviral technology, RNAi has been widely studied for its affect on FMDV (Chen et al. 2004; Kahana et al. 2004; Mohapatra et al. 2005). However, like other RNA viruses, FMDV is antigenically variable and undergoes rapid mutation. FMDV can easily escape the host immune system through the high variability of its surface antigens. Establishing RNAi as a viable approach against FMDV requires resolving at least one major issue (Gitlin and Andino 2003; Grubman and Santos 2005): the high genetic variability of the virus. RNAi directed to specific the gene sequence of certain FMDV strains may face risk, especially in the event of an emergent FMD outbreak, without any information concerning the serotype or genotype of the isolated pathogen although early protection is needed. Therefore, it may be necessary to design a multiple-siRNA expression system that focuses on several regions of the viral genome (Gitlin and Andino 2003; Nishitsuji et al. 2006; Song et al. 2003). DNA vector-based RNAi technology has been developed and has highlighted the general utility of RNAi against viruses in vivo (Sui et al. 2002). A dual siRNA expression system was established to generate two different siRNA molecules that specifically target two genes of HBV (Wu et al. 2005) simultaneously. This system has been extended to produce two siRNA duplexes simultaneously that target the S gene of HBV and the gp120 gene of HIV-1, respectively (Wu et al. 2007).

In this study, we constructed a plasmid system that can produce multiple siRNAs targeted to VP1 of the FMDV genome of serotype O, A and Asia I, respectively, and can cross-inhibit FMDV replication in BHK-21 cells and suckling mice.

Materials and methods

Cells, animals and viruses

BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (pH 7.4). Cultures were incubated at 37°C with 5% CO2. Suckling mice (C57BL/6), 2 to 3 days old and weighing 3 to 4 g, were maintained by Bio-pharmacy, Jinyu Group Co. LTD. FMDV types O (HKN/2002 [GenBank accession number AY317098]), FMDV types Asia I (YNBS/58 [GenBank accession number AY390432]), and one PRV isolate (Ea [GenBank accession number AY318876]) were used for viral challenge.

Construction of plasmids

The mouse U6 promoter (P_{U6}) was cloned into the Nde I/EcoR I sites of pcDNA3.1B(–) vector (Invitrogen, Groningen, The Netherlands), replacing the cytomegalovirus promoter in it, to generate the parent vector, pU6. As a general strategy for constructing siRNA-expressing plasmids, inverted repeats multiple-targeting the VP1 genes of 3 FMDV types (O, Asia I and A) were subcloned into pU6 under the control of the U6 promoter and termination signals of 4 or 5 thymidines (Fig. 1a). The plasmid, pCWN11, contains an inverted repeat corresponding to 23 nt, 29 nt and 27 nt of the VP1 cDNAs of serotype A, Asia I and O FMDV, respectively, and buffer sequences are used as the joints of the VP1 regions of three FMDV serotypes (Fig. 1a). As a control for nonspecific effects, we used plasmid, pLacZ, containing an inverted repeat corresponding to nt 1353 to 1435 of the β-galactosidase gene of *Escherichia coli*, which has no homology to the FMDV HKN/2002 genome, as confirmed by sequence analysis.

To analyze the silencing of the expression of the exogenous reporter gene, 3 reporter plasmids were constructed by cloning the VP1 cDNAs of 3 FMDV types into the EcoR I-BamH I sites of pEGFP-N1 (Clontech, Palo Alto, CA) to form fusion green fluorescent proteins, respectively. The resultant plasmids were pO1, pA5, and pAs9 (Fig. 1b). The correct open reading frames confirmed by sequencing retained the fluorescent properties of the fusion protein.

Transient cellular transfection and analysis of VP1 and EGFP expression in BHK-21 cells

Cell cultures were incubated at 37°C with 5% CO2. The day before transfection, cells were trypsinized, diluted with fresh medium, and seeded into 96-well culture plates. BHK-21 cells (about 80% confluent) were cotransfected with target plasmids (0.2 μg) and siRNA-expressing plasmids (0.1 μg) at a 2:1 ratio with Lipofectamine 2000 as described by the manufacturer. 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 1/4.5 s.

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 real-time quantitative RT-PCR (Q-RT-PCR) analysis using the SYBR RT-PCR Kit (Perfect Real Time) (TaKaRa, Kyoto, JP). 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

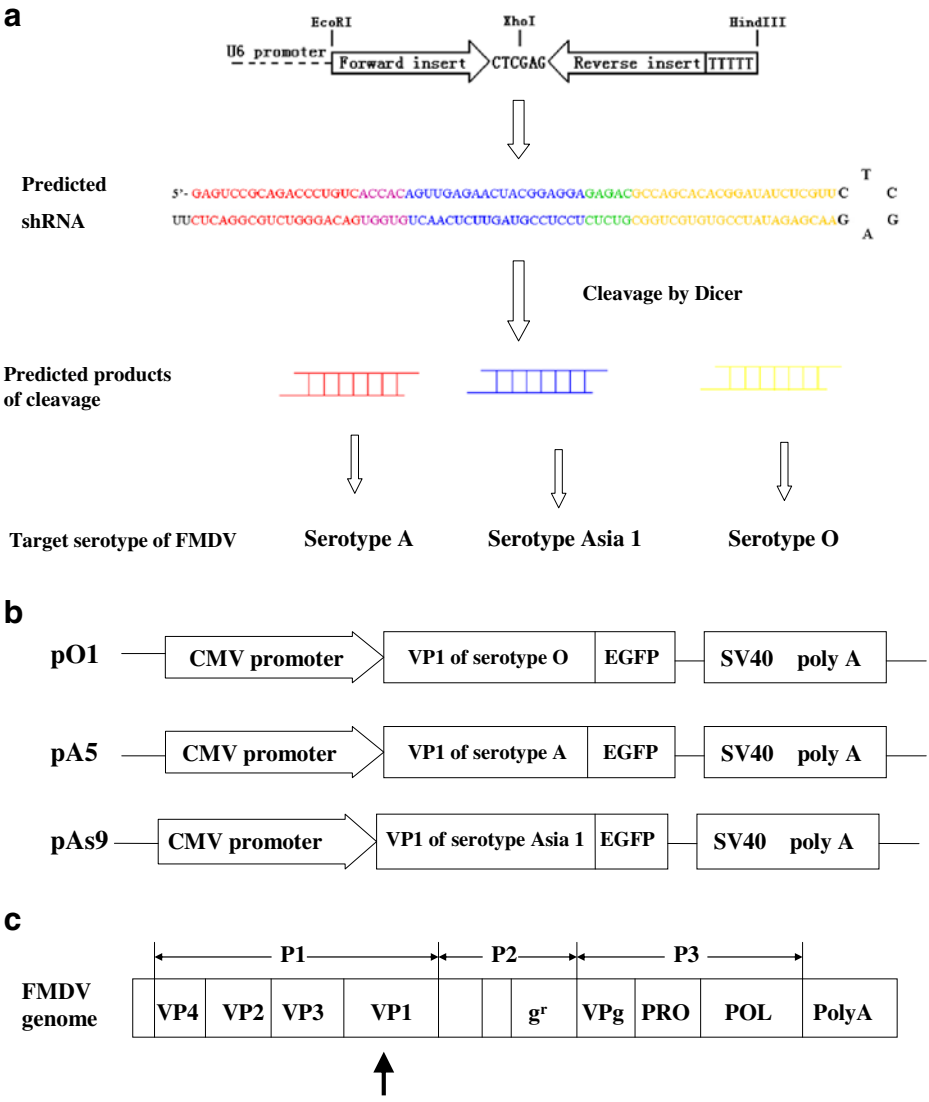

Fig. 1 Schematic representations of siRNA-expressing plasmids, target constructs, target viral mRNA, and predicted siRNAs. **a** An inverted repeat was inserted at the 3′ end of the mouse U6 promoter. The forward sequence of the repeat is 69 nt long, corresponding to the region of interest of the VP1 genes. Red, blue and yellow nucleotides represent the sequences corresponding to VP1 of FMDV serotype A, Asia I and O, respectively. Purple and green nucleotides are buffer sequences. Purple is the intersection of red and blue; green is the intersection of blue and yellow. Buffer sequences belong to the VP1 genes of adjacent serotypes, as the colors represent. The forward and reverse motifs are separated by a 6-nt spacer, 5′-CTCGAG-3′. The transcriptional termination signal of 5 Ts is added at the 3′ end of the inverted repeat. The synthesized RNA is predicted to fold back to form a hairpin dsRNA, which would be processed into the putative siRNAs. **b** To monitor the function of siRNAs, the VP1 cDNAs of three FMDV types were cloned into pEGFP-N1 respectively, as described in Materials and Methods. **c** The FMDV genome contains a unique open reading frame. The arrow at the bottom shows the site targeted by VP1-specific siRNAs

and displayed graphically by iCycler iQ Real-Time PCR Detection System Software Version 3.0A (BioRad, Hercules, CA). Beta-actin was used as a positive control. Relative mRNA levels of target genes are presented as the ratio of the target gene product quantity to 1 ng beta-actin (ng/1 ng beta-actin). Statistical analysis was performed with the Microsoft Excel program (Microsoft, Redmond, WA).

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 tissue culture infective dose ($TCID_{50}$) 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-expressing plasmid per well. After 5 h of transfection, the transfection complex was removed and the cells were washed twice with DMEM. The transfected cells in one well of the 96-well plates were then infected with 100 TCID50 of FMDV HKN/2002 or YNBS/58 per 0.1 ml. After 1 h of adsorption, the inoculum was removed and the cells were washed twice with DMEM. The infection then proceeded in DMEM supplemented with 10% fetal bovine serum. Cell supernatants were collected at different time postinfection (p.i.), and the virus titers were determined three times on BHK-21 cells. Statistical analysis was performed with the Microsoft Excel program (Microsoft, Redmond, WA).

Viral challenge assay in suckling mice

Suckling mice (6 groups of 10 each) were inoculated by subcutaneous injection in the neck with serially diluted viruses and then monitored for 5 days. The 50% lethal dose (LD_{50}) was estimated by the Reed-Muench method (Reed and Muench 1938). The suckling mice were subcutaneously injected in the neck with 100 or 200 μg siRNA-expressing plasmids dissolved in 100 μl of saline. After 6 h, the suckling mice were challenged with 20 or 100 LD_{50} of FMDV HKN/2002 or YNBS/58 per 0.1 ml by subcutaneous injection into the neck near the site which received the injected DNA and were observed for 5 days postchallenge. For detecting the therapeutic potential of siRNAs, some animals were treated with plasmid-virus complex and observed continually. Statistical analysis was performed with log rank test (http://bioinf.wehi.edu.au/software/russell/logrank/).

Results

The expression of VP1-EGFP fusion genes of FMDV serotype O, A and Asia I was reduced by the treatment with the multiple targeting siRNA-expressing plasmid

We constructed the multiple targeting siRNA-expressing plasmid, pCWN11, by using buffer sequences as the joints of the VP1 regions of 3 FMDV serotypes. To determine the influence of pCWN11 on the expression of FMDV VP1 in cultured cells, we cotransfected siRNA-expressing plasmids with either the target plasmid (pO1, pA5 and pAs9, respectively) or the control plasmid pEGFP-N1 into BHK-21 cells. After 24 h of incubation post-transfection, we subjected the cells to fluorescence microscopic analyses. Co-transfection of pCWN11 with any of 3 reporter plasmids resulted in a remarkable reduction in EGFP signal relative to the control, whereas the control construct (pLacZ) showed no

significant reduction of EGFP expression (Fig. 2a). In contrast, cotransfection of pEGFP-N1 with pCWN11 resulted in no significant reduction of EGFP expression compared with the control. To 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 (Fig. 2b). The level of fusion EGFP genes, as determined by Q-RT-PCR, was significantly decreased about 50% in cells transfected with pCWN11. The results indicate that the plasmid, pCWN11, could effectively and specifically inhibit the expression of FMDV VP1 in BHK-21 cells for serotype O, A and Asia I, respectively.

The replication of FMDV serotype O and Asia I was specifically inhibited by the multiple targeting siRNA-expressing plasmid, pCWN11, in BHK-21cells

BHK-21 has been extensively used for diagnosis and virus identification of FMDV (Dumbell et al. 1989). To test the antiviral activity of the siRNA-expressing plasmid pCWN11, transfected cells were infected with 100 50% tissue culture infective doses ($TCID_{50}$) of HKN2002 (FMDV serotype O) or YNBS/58 (FMDV serotype Asia I) at 24 h post-transfection. Cells were observed continuously under the microscope. The normal BHK-21 cells are fibroblastic, grow in a monolayer, and have a well-defined tendency to parallel orientation (Macpherson and Stoker 1962). Viral infection causes a marked cytopathic effect (CPE) ending in total cellular detachment, rounding up, and destruction, which can be observed by microscopy (Dumbell et al. 1989). Microscopy examination revealed that the CPE on infected cells was delayed when the BHK-21 cells were transfected with the siRNA-expressing plasmid pCWN11, relative to cells in the control groups (mock-transfection, not treated or transfected with a control plasmid, pLacZ, expressing *E. coli* β-galactosidase-specific shRNA). Viral replication of the unrelated PRV

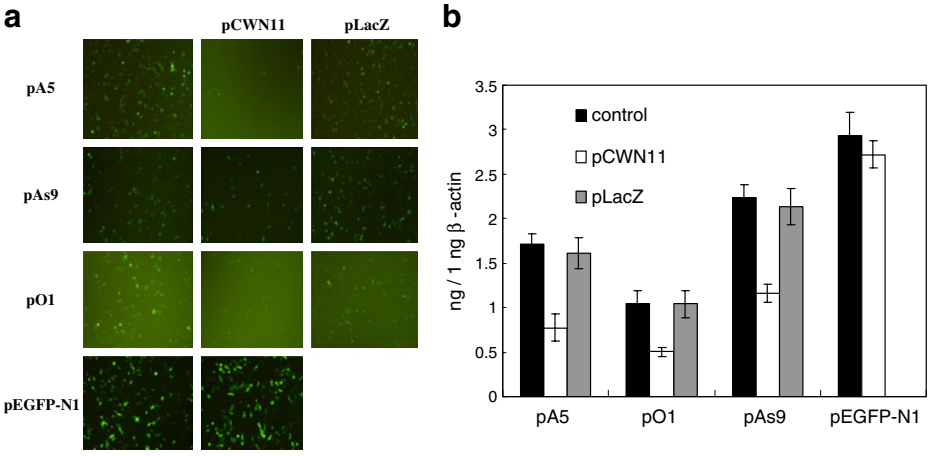

Fig. 2 Multiple targeting siRNAs-expressing plasmid pCWN11 inhibited specifically the expression of FMDV VP1 of three serotypes in BHK–21 cells respectively. **a** Fluorescence micrographs of cells transfected with reporter plasmids (pA5, pAs9 and pO1) and cotransfected with either pCWN11 or pLacZ. As controls for nonspecific effects, cells were co-transfected with pEGFP–N1 and pCWN11. At 24 h after transfection, representative fields were photographed. **b** Real-time Q-RT-PCR analysis for the expression of EGFP. Relative mRNA levels of EGFP are presented as the ratio of the EGFP mRNA quantity and the 1 ng β-actin (ng/1 ng β-actin) to facilitate comparison with the levels of inhibition to different VP1. Data is shown as the average of three repeat experiments. Error bars refer to standard deviations

isolate Ea was not significantly altered in BHK-21 cells transfected with either pCWN11 or the control plasmid, pLacZ, suggesting that the antiviral effect mediated by siRNAs is efficient and highly sequence specific.

To investigate the effect of pCWN11 on FMDV replication, the $TCID_{50}$ of supernatants of cell lysates made at intervals post-challenge were measured. In agreement with the microscopic observations, the supernatant $TCID_{50}$ for HKN/2002 of the cells transfected with pCWN11 was reduced. About 10^3 $TCID_{50}$ of HKN/2002 virus progeny was detected in supernatants collected from cells transfected with pCWN11 at 19 h p.i., whereas over 10^5 $TCID_{50}$ of virus was determined in supernatants collected from control cells (Fig. 3a). In the case of challenge with YNBS/58, virus yield in cells examined at 16, 22, and 42 h p.i., decreased by 10- to 100-fold in cells pretreated with pCWN11, but there was no significant decrease in control cells (Fig. 3b). As expected, pCWN11 gave no marked inhibition to PRV at any time point assayed (Fig. 3c).

The multiple targeting siRNA-expressing plasmid, pCWN11, induced cross-resistance to heterologous FMDV infection in suckling mice

To further test whether the multiple targeting siRNA-expressing plasmid, pCWN11, could inhibit the replication of heterologous FMDV isolates, the HKN/2002 strain of FMDV serotype O and the YNBS/58 strain of FMDV serotype Asia I were employed to perform viral challenge in suckling mice. We challenged suckling mice pretreated by subcutaneous injection of siRNA-expressing plasmids in the neck or by of injection with plasmid-virus

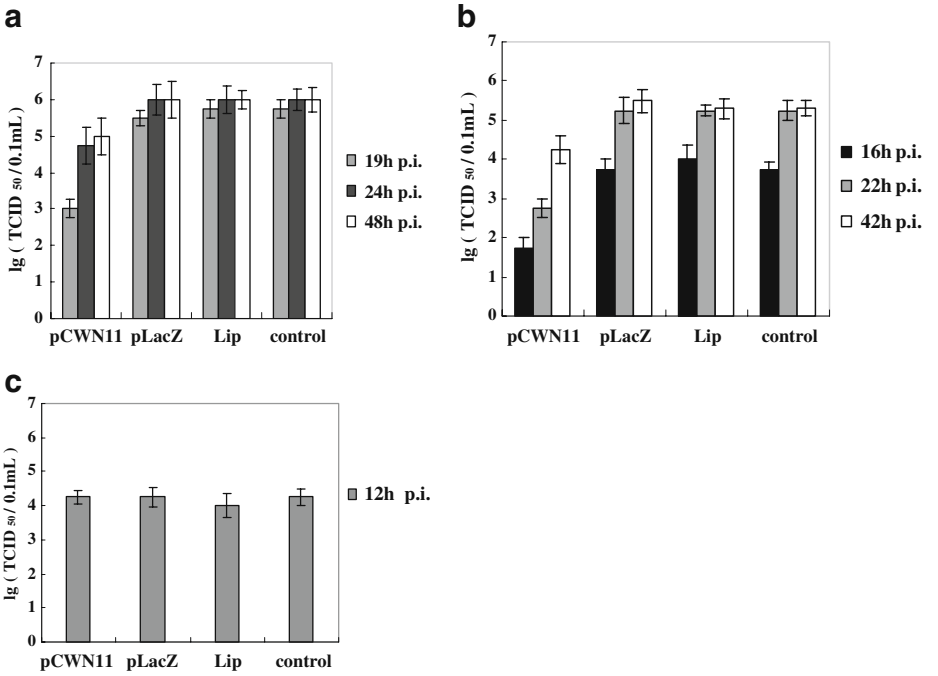

Fig. 3 Decrease of virus yield in BHK-21 cells transfected with the multiple targeting plasmid, pCWN11. Cells transfected with either pCWN11 or the control plasmid, pLacZ, were infected with 100 $TCID_{50}$ of FMDV HKN/2002 (**a**), FMDV YNBS/58 (**b**) or PRV Ea (**c**) per 0.1 ml. Culture supernatants were collected at different times, and the virus titer ($TCID_{50}$) was determined three times on BHK-21 cells. Error bars indicate standard deviations

complex. All PBS-treated mice died within 60 h after 20 LD_{50} of either HNK/2002 or YNBS/58 challenge, and animals treated with the control plasmid, pLacZ, which was predicted to synthesize a shRNA heterologous to the FMDV genome, were not protected at all (log-rank test, P=0.836 and P=0.759, respectively) (Figs. 4b and 5b).

In YNBS/58 challenge experiments, 10 of 40 mice treated with pCWN11-FMDV complex were protected (Fig. 4a). However, 18 of 37 mice pretreated with pCWN11 survived a viral challenge of 20 LD_{50} for 5 days of observation (Fig. 4b). In addition, animals pretreated with pCWN11 but challenged with 100 LD_{50} of YNBS/58 also had reduced susceptibility to virus infection, and the antiviral effects induced by either 100 or 200 μg of pCWN11 were similar. All mice of control groups died within 48 h after YNBS/58 challenge of 100 LD_{50}. The difference between the survivals of mice treated with pCWN11 and the PBS control was statistically significant (P<0.00001) (Fig. 4c and d).

In the case of challenge with 20 LD_{50} HKN/2002, 4 of 38 mice treated with pCWN11-FMDV complex and 12 of 40 mice pretreated with pCWN11 survived, whereas a significant difference in mouse survival was also seen between mice treated with pCWN11 and controls (P <0.00001) (Fig. 5). Furthermore, in both HNK/2002 and YNBS/58 challenging assays, the time of 50% death of mice was delayed for 12 to 24 h in groups treated with, pCWN11, as compared

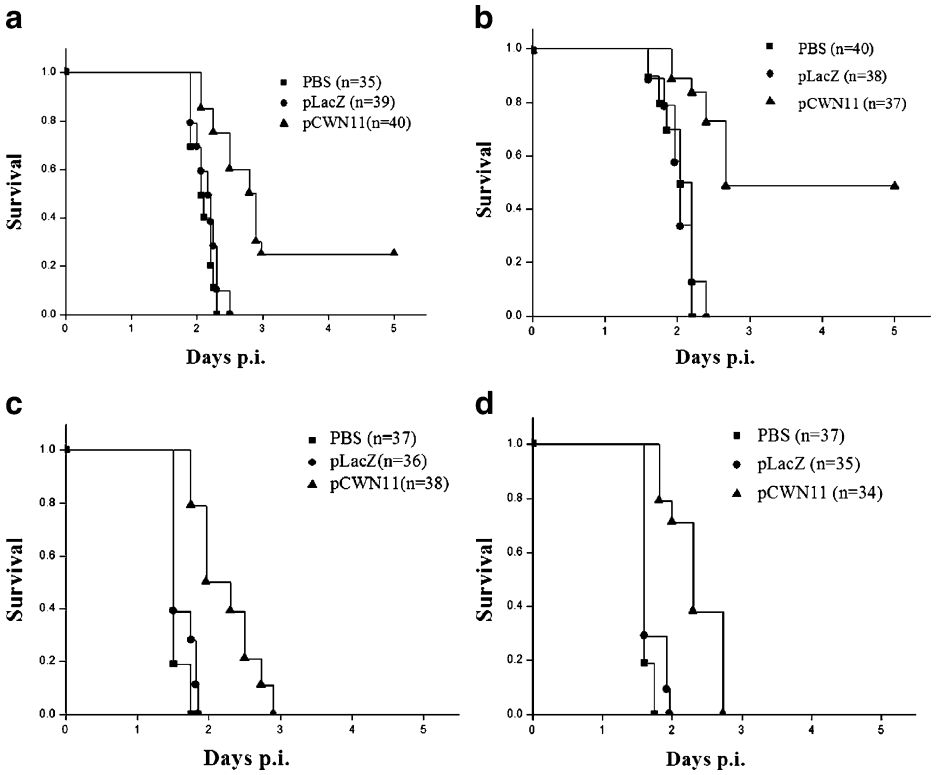

Fig. 4 Plasmid, pCWN11, expressing siRNAs in vivo induce an antiviral response in suckling mice and promote survival after FMDV YNBS/58 infection. Suckling mice were challenged by subcutaneous injection in the neck with 20 LD_{50} of YNBS/58 after 0 h (**a**) or 6 h (**b**) of treatment with 100 μg plasmids. Alternatively, suckling mice were challenged by subcutaneous injection in the neck with 100 LD_{50} of YNBS/58 after 6 h of treatment with 100 μg plasmids (**c**) or 200 μg plasmids (**d**). All animals were observed for 5 days after challenge

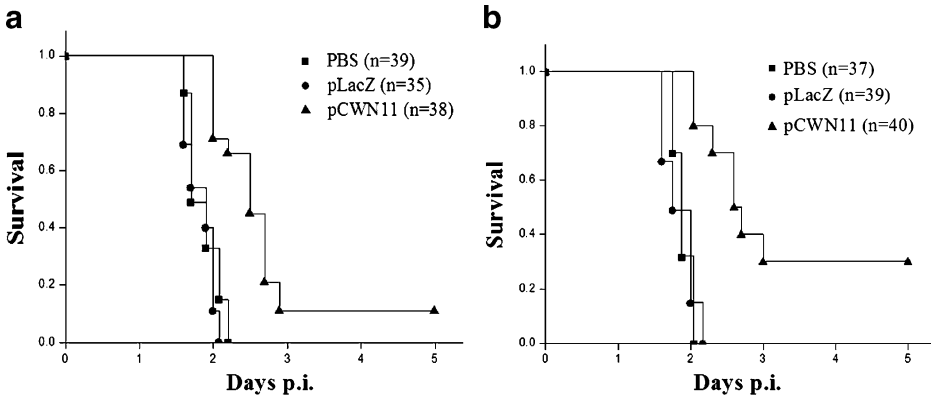

Fig. 5 pCWN11 expressing siRNAs in vivo induces an antiviral response in suckling mice after FMDV HKN/2002 infection. Suckling mice were challenged by subcutaneous injection in the neck with plasmid (100 μg) –virus complex containing 20 LD_{50} of HKN/2002 (**a**) or challenged with 20 LD_{50} of HKN/2002 after 6 h of treatment with siRNA-expressing plasmids (**b**). All animals were observed for 5 days after challenge

with control groups. Internal organs from the surviving mice appeared normal when the animals were killed at the end of the observation period. All dead mice had extensive damage to the viscera, indicating massive virus replication in tested animals.

Discussion

FMDV has seven distinct serotypes characterized by serological analysis. Viruses are likely to evolve mechanisms to suppress or escape an RNAi response. Long-term silencing of viral protein expression by siRNAs has been reported to result in the emergence of viruses resistant to RNA interference (Das et al. 2004; Gitlin et al. 2005). This issue needs to be addressed if siRNAs are to be used therapeutically. Resistance was caused by the generation of mutations in the siRNA target site. Focusing on the conserved regions of the viral genome (Chang et al. 2005; Dave and Pomerantz 2004) or simultaneously targeting several viral sequences (Chang et al. 2005; Gitlin et al. 2005; Geisbert et al. 2006) may be an approach to resolve this issue. Our strategies to address these potential problems are to generate multiple siRNA molecules that can target different sites or genes on the viral genome. The previous study demonstrated significant cross-inhibition of heterologous FMDV by multiple siRNAs targeting various conserved regions of the viral genome, including a long 5′ non-coding region (5′ NCR), a shorter 3′ non-coding region (3′ NCR), a small covalently bound virus-encoded protein (VPg), a viral polymerase (POL), and a viral capside protein (VP4) (Liu et al. 2005).

In this study, we constructed a multiple-siRNA expressing plasmid pCWN11. This siRNA-expressing plasmid carried three independent siRNAs that target the VP1 genes of serotype A, O and Asia I of FMDV. To determine whether this system can be used to inhibit gene expression of the three target genes, the multiple-siRNA expressing plasmid was co-transfected with either of target plasmids. The data revealed that co-transfection resulted in a significant reduction in the corresponding viral transcripts. Expression of an 82-nt siRNA heterologous to the FMDV genome did not show a significant reduction of VP1 mRNA levels. In addition, RNAi action was abolished when targeted to a heterologous EGFP gene, suggesting that siRNAs mediated significant reductions in the levels of a specific target mRNA, and not a global down-regulation resulting from activation of the dsRNA-activated

protein kinase R, which could lead to an inhibition of protein translation in a nonsequence-specific manner. Here, we report that a pool of three siRNAs synthesized in vivo dramatically inhibit viral replication in cultured cells that are susceptible to FMDV of either serotype O or serotype Asia I, and the inhibition does not extend to an unrelated PRV. Suckling mice treated with the siRNA-expressing plasmid have significantly reduced susceptibility to both serotype O and serotype Asia I of FMDV. Although it was not determined whether the system could inhibit the replication of FMDV serotype A, we suggest that the system has the potential for this activity due to the significant reduction of the fusion green fluorescent protein of serotype A reporter plasmid when co-transfected in cells. All of the present results indicate that this system had significant inhibitory effects on viral mRNA expression and the replication of several FMDV serotypes.

Previous work by Wu et al. (2005) established a dual siRNA expression system that could simultaneously express two different siRNA molecules to target two genes specifically. The system carried two independent siRNA expression cassettes, and each of cassettes had one U6 promoter. This dual siRNA system was applied to express two different 21-bp hairpin siRNA duplexes simultaneously that specifically attack the HBs and HBx genes of HBV, respectively. The aim of this study is to design a multiple-siRNA expressing system to inhibit the replication of several FMDV serotypes. In order to construct the system, we choose buffer sequences to fusing the targeted DNAs together to form an inverted motif. The synthesized RNA is predicted to fold back to form a hairpin dsRNA, which would be processed into the putative tripartite siRNAs by Dicer in vivo. Therefore, the multiple-siRNA expressing system containing only one U6 promoter was established. Buffer sequences allow the use of DNA templates to synthesize a pool of three active siRNAs under the control of an RNA polymerase III (Pol III) promoter synthesized in vivo. Tuschl and colleagues (Elbashir et al. 2001b) defined the active, in vitro synthesized siRNA as a 21-nt-long dsRNA with symmetrical 2- to 3-nt 3′ overhangs. In other organisms such as *C. elegans* and *Drosophila*, the input RNA can be either in the form of a long dsRNA or a hairpin dsRNA (Kennerdell and Carthew 2000; Tavernarakis et al. 2000). Presumably, both forms of RNA are further cleaved by Dicer, an RNase III enzyme, to generate 21- to 23-nt-long siRNA (Bernstein et al. 2001a; Knight and Bass 2001). The resulting 21- to 23-nt siRNA mediates degradation of the complementary homologous RNA (Bernstein et al. 2001b; Sharp 2001). Some siRNAs can discriminate between mRNAs that differ by only a single nucleotide (Ding et al. 2003; Phipps et al. 2004), but genome-wide assessments of siRNA specificity suggest that mRNAs with only partial complementarity to a siRNA can also be targeted for destruction (Jackson et al. 2003). Although not all of the mismatches are indispensable to effective target cleavage, complementarity between siRNA and their targets would be expected. Although the exact process of designed RNA cleavage by Dicer is unknown, we expect the buffer sequences can reduce the mismatch without increasing the length of dsRNA. This prediction is based on the fact that Dicer preferentially cleaves dsRNAs at their termini (Zhang et al. 2002). The cleavage at the buffer sequences had no effect on the accuracy of siRNA. If template sequences were concatenated end to end under the control of one promoter without buffer sequences, there would occur a deviation between the synthesized siRNA and the expected siRNA when the hairpin double stranded RNA (dsRNA) were cleaved by Dicer. The deviation would tend to have a ripple effect on the accuracy of siRNA corresponding to target sequences by synthesizing a pool of siRNAs containing several mismatch base pairs. To a certain extent, augmenting the template length can avoid the ripple effect cause by the deviation, but we can not confirm the affect of excess length of dsRNA.

Our results suggest that the multiple-siRNA expressing system is a feasible strategy to overcome the issue of high genetic variability of FMDV and viral escape. This approach may used to deal with several viruses, which are especially useful in the treatment of co-infections by multi-pathogens. In addition, the application of siRNA therapy in viral infections would be beneficial especially to those viruses with a high mutation rate.

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