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Short Hairpin RNA Targeted to the Highly Conserved Regions of *L* and *3D* Gene Inhibits Replication of Foot-and-Mouth Disease Virus *in vitro*

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Abstract: Foot-and-Mouth Disease Virus (FMDV) is a highly contagious vesicular disease affecting >33 species of cloven-hoofed animals. Because of the limited effectiveness of existing antiviral approaches including vaccines and antiviral drugs, new strategies for controlling FMD outbreak are needed. RNA interference (RNAi) is considered to be one of the powerful emerging approaches to inhibit virus replication in mammalian cells. In this study, two short-hairpin RNA (shRNAs) expressing vectors (pShuttle-shRNA-L and pShuttle-shRNA-3D) targeting to the L and 3D non-structural protein coding regions of FMDV were employed to evaluate their antiviral ability in Baby Hamster Kidney cells (BHK-21). Results demonstrated that transfection of shRNA-expressing plasmids gave a significant weakened Cytopathic Effect (CPE) as well as a manifest decreased viral tilters and amount of FMDV mRNA copy numbers compared with the control cells post challenge of O/HK/2001. In this study, the data indicates that RNAi may be a potential therapeutic approach to limit the spread of FMDV in livestock.

Key words: Foot-and-mouth disease virus, RNA interference, shRNA, L and 3D non-structural protein coding region, live stock, China

INTRODUCTION

Foot-and-Mouth Disease (FMD) is an acute and highly contagious disease that affected >33 species of cloven-hoofed animals such as cattle, swine and sheep. Because of its rapid spread and requires socioeconomic consideration, it is listed by the World Organisation of Animal Health (Brown, 2003). The causative agent is Foot-and-Mouth Disease Virus (FMDV) which belongs to the genus Aphthovirus of the family Picornaviridae (Domingo and Escarmis, 2003) characterized by its highly potential genetic and antigenic variation which led to the classification of seven serotypes of FMDV (A, O, C, Asia1, SAT1-SAT3) on the basis of their ability to induce cross-protection in animals (). The viral partical contains a positive-strand of about 8500 nucleotides (nt), containing a unique long Open Reading Frame (ORF) encoding a polypeptide that is cleaved into four structural proteins (VP1-VP4) and eight non-structural proteins (L, 2A-2C, 3A-3D) by viral proteases (Feng *et al*., 2004). Current measures for controlling of FMD outbreak including routine vaccination and slaughtering of infected and contact animals. Routine vaccination is to some extent valid in preventing the disease (Chen *et al*., 2004).

Nevertheless, FMDV replicates so rapidly that significant clinical symptoms can appear as early as 2 days post-exposure to FMDV and moreover complete protection can not been induced prior to 7 days post-inoculation (Santos *et al*., 2005). What's more, vaccines based on inactivated virus present the risks of incomplete inactivation, not to mention the existing seven different virus serotypes and numerous subtypes (Kahana *et al*., 2004). So, the use of current FMD vaccines to induce early protection is limited. The spreading capacity of FMDV and its high rate of mutation, diverse host range, low infectious dose requirement and multiple routes of transmission make it a real threat to the beef and dairy industries in many countries (Liu *et al*., 2005; Kahana *et al*., 2004).

Therefore, it is fiercely urgent to develop novel rapid-acting antiviral approaches that could effectively inhibit FMDV replication in outbreak situations. RNA interference (RNAi) is a process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by 21-23nt small interfering RNA (siRNA) that is homologous in sequence to the silenced gene (Fire *et al*., 1998; Elbashir *et al*., 2001; Zamore, 2001). As a cellular defense mechanism, RNAi can be used to regulate gene

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expression (McCaffrey *et al*., 2003). Now-a-days, inhibition of virus replication by RNA interference has been reported for a number of human disease viruses including human immunodeficiency virus (Kitabwalla and Ruprecht, 2002; Stevenson, 2003; Bennasser *et al*., 2007), hepatitis A-C virus (McCaffrey *et al*., 2003; Kanda *et al*., 2004; Shlomai and Shaul, 2004; Henry *et al*., 2006) and other diseases.

Inhibition of FMDV replication via RNAi has also been reported recently. A plasmid expressing shRNA targeting VP1 region (Chen *et al*., 2004), adenovirus vector expressing shRNA targeting 1D and 3D region (Chen *et al*., 2006), adenovirus mediated shRNA corresponding to the 2B and 3C region (Kim *et al*., 2008, 2010) have been shown to possess activity against FMDV *in vitro* and *in vivo*; the antiviral effect of various siRNAs on virus replication was investigated both before and after viral infection (Kim *et al*., 2008; Levy *et al*., 2009). RNAi therefore, opens up a possible new era in suppressing virus replication. In this pilot research, researchers constructed two expression plasmids delivering shRNAs designed from highly conserved regions of *L* and *3D* gene of FMDV and tested multiple shRNA candidates by conforming sequence-specific inhibition in independent EGFP reporter assays. Researchers demonstrated that both of these shRNAs were validated to inhibit FMDV replication in BHK-21 cells and the antiviral effect was dose-dependent. However, the mixture of shRNAs did not present a significant increased inhibitory ability. The results of this study suggest that treatment with shRNAs may be helpful in the control of FMD in an outbreak.

MATERIALS AND METHODS

Cell and virus: Baby Hamster Kidney cells (BHK-21) were used to grow FMDV and to determine virus titer ($TCID_{50}$). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS; PH7.4). Cultures were incubated at 37°C with 5% CO_2. FMDV isolate O/HK/2001 (Chen *et al*., 2006) is used for viral challenge.

Design and construction of plasmid expressing short hairpin RNAs: Conserved target sequences were selected from FMDV O/HK/2001 based on the alignment of sequences of 7 serotypes of FMDV genome which were obtained from the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). The chosen sequences were nt 1377-1395 of the L protein coding region and nt 7867-7885 of the 3D protein coding region. Two 59-nt long annealed oligonucleotides with overhanging ends were cloned into BamHI/EcoRI sites of pSIREN-Shuttle vector driven by the U6 promoter (Clontech, Palo Alto, CA). The oligonucleotides contained a 5-nt overhanging BamHI site (GATCC) plus an added G followed by a 19-nt sense siRNA sequence (L: ACACCGGAATCGGCTCCGC;3D:GTTGATCTCCGTG GCAGGA), a 9-nt loop (TTCAAGAGA), a 19-nt reverse complementary antisense siRNA sequence (L: GCGGTGC CGATTCCGGTGT;3D:ATCCTGCCACGGAGATCAAC), an added C and 7 Ts as RNA polymerase III termination signal. The resultant recombinant plasmids were pShuttle-shRNA-L and pShuttle-shRNA-3D. The resulting RNA transcripts were expected to fold back and form a stem-loop structure with a 19-nt region homologous to the target sequences of the FMDV genome.

Construction of reporter plasmids: To analyze the silencing ability of the expression of exogenous reporter genes, two reporter plasmids were constructed by cloning the two target sequences of FMDV genome into the EcoRI/BamHI sites of pEGFP-C1 (Clontech, Palo Alto, CA) to form fusion green fluorescent proteins. The resultant recombinant plasmids were pEGFP-L and pEGFP-3D. The two target sequences were amplified by RT-PCR using FMDV genome as template.

Primers targeting L region were sense 5'-CGGAA TTCCATACCCAACAACCACGA-3' and antisense 5'-CGCGGATCCCAGATTGGTTTTGTGAT-3'primers for amplifying and sequencing 3D region were sense 5'-CGGAATTCCAGACCCAACAACCACGA-3' and antisense 5'-CGCGGATCCCAGATTGGTTTTGTGAT-3'.

Cell transfections and GFP expression: The day before transfection, BHK-21 cells of 2×10^5 were plated in each well of 24 well plates. After day, 90% confluence of BHK-21were present at the time of transfection. Cells were transfected with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol in the presence of 1.8 μg shRNA-expressing plasmids and/or 0.6 μg recombinant reporter plasmids. After 6 h incubation, the transfection complex was removed and cells were washed twice with DMEM. A 24 h following the transfection, the cells were examined microscopically for GFP expression. Images were collected with a Nikon E950 video camera at a magnification of 40× with an exposure time of 1/8 sec.

Cell transfection and viral challenge: FMDV of 10^6 $TCID_{50}$ /0.1 mL was used for viral challenge. BHK-21 cells were transiently transfected with about 0.7 μg shRNA-expressing plasmids per well in 24 well plates. After 12 h incubation, the transfected BHK-21 cells were then infected immediately with 0.1 mL 100 $TCID_{50}$ FMDV. After 1 h absorption at 37°C, the suspension was removed and the cells were washed twice with DMEM. The inocula

were then proceeded in DMEM supplemented with 2% FBS. Supernatants were collected at 12, 24, 48 and 72 h following FMDV infection and were used in the kinetic analysis of FMDV.

Analysis of FMDV replication in BHK-21 cells: To assay the effect of shRNA on FMDV replication, quantitative real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and virus titrations were carried out. FMDV RNA copy numbers were determined by quantitative real-time RT-PCR. Real time RT-PCR was conducted using a one step PrimeScript™ RT-PCR kit (TaKaRa). Viral RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacture's introduction.

All assays were performed according to the manufactrurers' instructions. The probe was: 5'-TGAAA GCAAGAGCCCCGGTCGT C-3' with 5'end labeled with 6-FAM and the 3'end labeled with TAMRA. The primers that flank the TaqMAN probe site amplify 85 bp fragment targeting to the 3B region were: sense 5'-CGGT CCGATGGAGAGACAGA-3' and antisense 5'-CTTCACC GGTCCTTCATAAGGT-3'. Reaction was carried out under the condition of 40°C for 25 min, 94°C for 3 min followed by 40 cycles of 93°C for 45 sec and 55°C for 45 sec. The virus titer ($TCID_{50}$) of each supernatant was determined on BHK-21 cells.

RESULTS

shRNAs induces potent specific inhibition of FMDV replication in BHK-21 cells: To evaluate the specific inhibition of shRNAs in BHK-21 cells, researchers constructed two plasmids expressing candidate shRNAs and two EGFP reporter plasmids. The shRNA-expressing plasmids were co-transfected into BHK-21 cells with either the homologous or the heterologous reporter plasmids or the control plasmid pEGFP-C1. A 24 h incubation post transfection, the cells were subjected to fluorescence microscopic analysis (Fig. 1a-f).

Results showed that GFP expressing was obviously decreased in cells transfected with homologous shRNA-expressing plasmids (Fig. 1d, e) compared with cells transfected with heterologous shRNA-expressing plasmids (Fig. 1c) or non-transfected cells (Fig. 1b) whereas the control constructs (pEGFP-C1) gave the significant expression of GFP (Fig. 1a). No fluorescence was seen in blank control cells (Fig. 1f). The level of inhibition slippered a little from 24-48 h post transfection but at 72 h post transfection, the inhibitory effect dropped. The ability of inhibition mediated by shRNA-L was generally better than that of shRNA-3D. The results primarily suggested that the shRNAs could effectively and specifically inhibit the expression of FMDV L and 3D gene in BHK-21 cells.

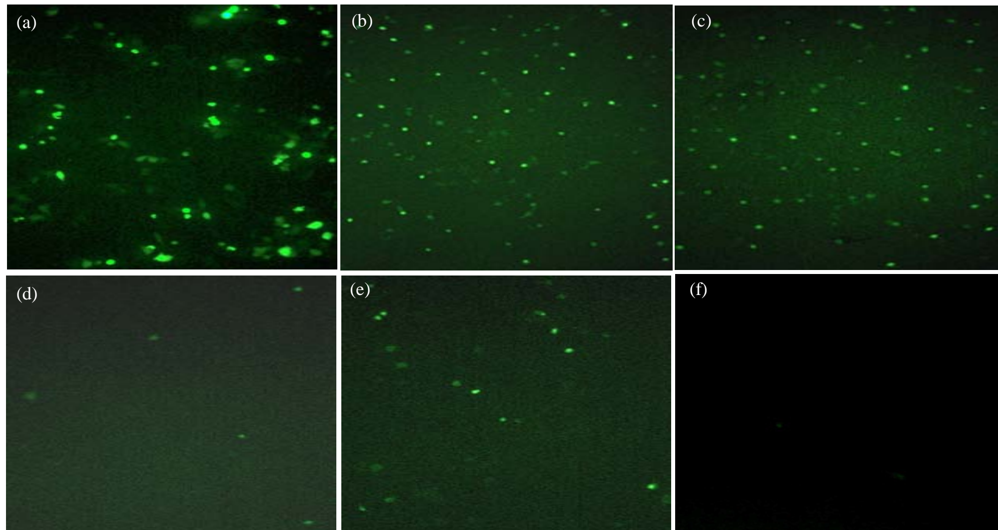

Fig. 1: Fluorescence micrographs of cells transfected with reporter plasmids and cotransfected with either the corresponding or non-corresponding shRNA. A 24 h post transfection, representative fields were photographed (200x). (A)~(F) BHK-21 cells were (co-) transfected with plasmid pEGFP-C1 (control plasmid), pEGFP-L, pShuttle-shRNA-3D and pEGFP-L (heterologous shRNA-expressing plasmid), pShuttle-shRNA-L and pEGFP-L, pShuttle-shRNA-3D and pEGFP-3D (homologous siRNA-expressing plasmid), Lipofectamine 2000 reagent (Invitrogen), respectively

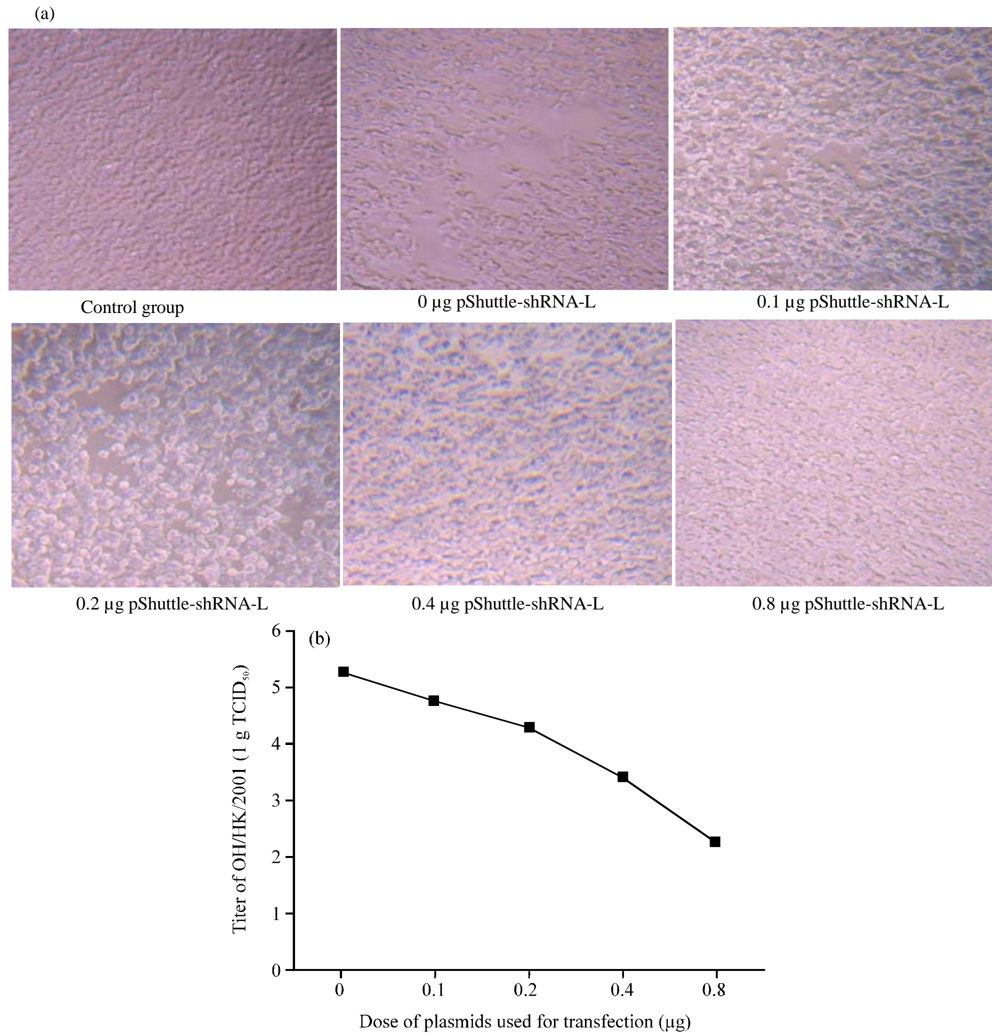

Fig. 2: a) Decrease of CPEs and virus yield in BHK-21 cells transfected with gradually dose of shRNA. Cells were inculated with pShuttle-shRNA-L at the concentration of 0, 0.1, 0.2, 0.4, 0.8 µg and challenged with 100 $TCID_{50}$ of FMDV O/HK/2001, respectively 12 h post inoculation, 24 h following FMDV challenge, cells were visualized with microscope and representive bright-field images were photographed (100x); b) Meanwhile, samples of supernatant were taken at 12, 24, 48 and 72 h following FMDV infection and virus titers ($TCID_{50}$) were determined in BHK-21 cells

Dose-dependent inhibition of FMDV replication by shRNAs: To determine whether the inhibition ability is dose-dependent, BHK-21 cells in 24 well plates were transfected with pShuttle-shRNA-L at four different concentrations: 0, 0.1, 0.2, 0.4 and 0.8 µg well^{-1}. A 12 h post-transfecion, cells were infected with 100 $TCID_{50}$ of FMDV except the control group. CPE of BHK-21 cells in each group were observed under the microscope 24 h p.i. (Fig. 2a). Results showed that the silencing effect almost vanished completely if shRNA concentration was diminished to 0.1 µg well^{-1} and transfection at low 0.2 µg well^{-1} gave rise to only a slight inhibition of FMDV replication as compared to non-transfected cells. Similarly, with the increasing of siRNAs dose, the evident inhibition of FMDV replication was observed. Enhanced silencing effect was observed when the transfected dose was 0.8 µg well^{-1} which led to an almost complete loss of CPE. The effect of RNAi on FMDV replication was further substantiated by measuring the $TCID_{50}$ of supernatant 24 h p.i. (Fig. 2b). Consistent with the observation when the BHK-21 cell transfected with gradually higher dose of shRNAs, $TCID_{50}$ of supernatants of cell lysates was reduced by approximately 4 ten fold dilutions compared with the control group. This result demonstrated a dose-response relation between siRNAs used for transfection and the efficacy of gene silencing.

Inhibition ability in BHK-21 cells by RNAi mediated by plasmid expressing shRNA: The antiviral effect of shRNAs on the replication of FMDV in BHK-21 cells was tested in 24 well plates, using 0.8 µg of each plasmid. Cells transfected with pSIREN-Shuttle vector served as control. About 24 h post transfection, all cells were infected with 100 $TCID_{50}$ of O/HK/2001. The CPE of BHK-21 cells were examined microscopically at 24 h p.i. Normal cells free of FMDV challenge grow as monolayers and have a pronounced tendency to adopt a parallel orientation. Viral infection causes a marked CPE resulting in complete cellular detachment, rounding up and destruction in the cells of the control groups. Whereas, the transfected group with shRNA-expressing plasmids (either pShuttle-shRNA-L or pShuttle-shRNA-3D) had a delay in the development of the characteristic CPE post challenge indicating that cells transfected with shRNAs could to some extent, reduce susceptibility to FMDV infection. $TCID_{50}$ of supernatants of cell lysates were measured at intervals post challenge, respectively. As shown in Fig. 3a, roughly 1 $TCID_{50}$ of O/HK/2001 virus progeny was detected in supernatant collected from pShuttle-shRNA-L transfected cells at 12 h p.i. which was the lowest than that in other groups whereas >100 $TCID_{50}$ of virus was determined in supernatants derived from control cells. The antiviral effects triggered by either pShuttle-shRNA-L or pShuttle-shRNA-3D were nearly similar at 24 h p.i. and extended to around 48 h p.i. No significant inhibition was observed 72 h p.i., excluding pShuttle-shRNA-L transfected cells in which virus titer turned out to be about 3.7 ten fold dilutions decreased compared to that of control cells.

To further demonstrate the levels of inhibition, Quantitative RT-PCR (Q-RT-PCR) was also been used to analyse the amount of FMDV RNA copy numbers at intervals post challenges. In agreement with the results of virus titer ($TCID_{50}$), the level of FMDV RNA monitored by Q-RT-PCR was evidently collapsed in cells transfected with homologous siRNAs as well comparing to that in control cells (Fig. 3). In particular, pShuttle-siRNA-L was remarkably effective which showed almost 99% viral inhibition at 12 h p.i. and endured to 24 h p.i. ($p<0.01$, ANOVA). On the other hand, pShuttle-siRNA-3D also displayed apparent ability of inhibition in the neighborhood of 90% ($p<0.05$, ANOVA). Although, the copy number of FMDV RNA accumulated continuously in the following hours (48-72 h p.i.), the mean difference were persistently significant, relative to that of control cells at the 0.05 level.

As an additional assay, researchers investigated whether it might be possible to suppress the replication of FMDV by two different shRNA-expressing plasmids

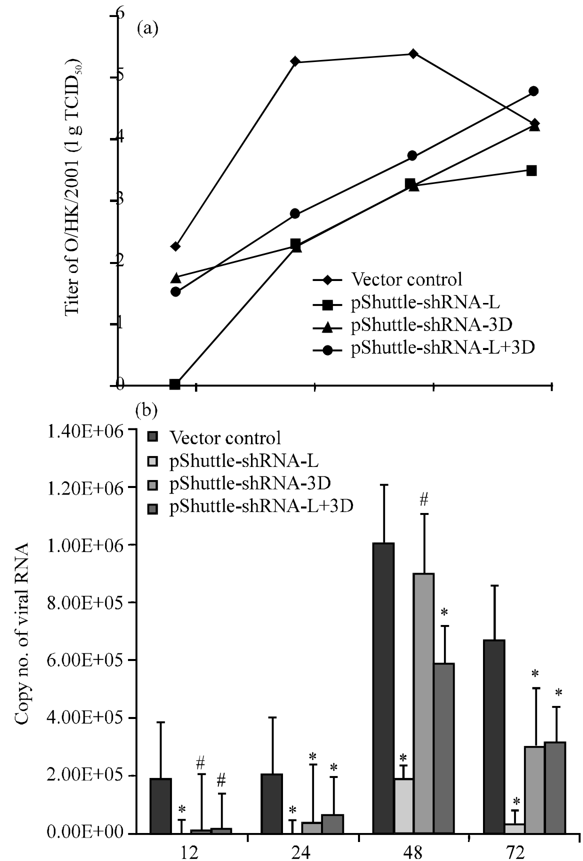

Fig. 3: a) FMDV specific shRNAs effectively protects BHK-21 cells from O/HK/2001 infection. Cells were inculated with 0.8 µg of each shRNA and with a mixture of both shRNAs at equal dose. Vector plasmid (pSIREN-Shuttle) was used as control. A 12 h post transfection, all cells were infected with 100 $TCID_{50}$ of FMDV. Samples of supernatants were collected after various time intervals following FMDV challenge. Virus titers ($TCID_{50}$) were determined in BHK-21 cells; b) the amount of FMDV RNA was determined by quantative real-time RT-PCR. The symbols # and * indicate p-values <0.05 and <0.01, respectively. Error bars indicate Standard Deviation (SD). Values are shown as the mean±SD and compared with pSIREN-Shuttle vector control groups

simultaneously. Equal amount of shRNA (0.4 µg pShuttle-shRNA-L mixed with 0.4 µg pShuttle-shRNA-3D) were cotransfected into BHK-21 cells and carried out the same procedures. As can be shown in Fig. 3a, virus titer generally higher in any time point than that of either pShuttle-shRNA-L or pShuttle-shRNA-3D transfected cells. FMDV RNA copy numbers transfected with the

mixtures was also significantly reduced measured by Q-RT-PCR but did not present any synergistic effect of inhibiting FMDV replication (Fig. 3b).

DISCUSSION

The FMDV genome is a positive-sense, single-stranded RNA that functions as both a messenger and replication template (Kanda *et al*., 2004), making it an attractive target for the study of RNAi. Thus, the use of siRNA corresponding to FMDV genes can theoretically be a powerful tool to silence its gene expression post-transcriptionally and may be a potential alternative strategy for viral inhibition. Nevertheless concerns like genetic variability of the pathogen, the large number of target species need to be addressed along with the therapeutic vaccines for effective control of FMD (Bayry and Tough, 2005; Joyappa *et al*., 2009). The present researches have investigated the effectiveness of using the specific shRNA expression-based vector targeting to conserved regions of L and 3D of the FMDV genome to inhibit FMDV replication. The results are consistent with previous studies that RNAi showed the capability of gene silencing in preventing FMDV infections (Chen *et al*., 2003, 2004; Santos *et al*., 2005).

One drawback of siRNAs have is that their effects are transient as mammalian cells apparently lack the mechanisms that amplify silencing in worms and plants to deal with this problem. A preferred approach is to apply vectors to express short hairpin RNA-essentially the double-stranded folded stem-looped-RNAs (shRNAs) (Karagiannis and El-Osta, 2005). Short hairpin RNA (shRNAs) can be transcribed when recombinant plasmids introduced into mammalian cells (Cullen, 2002). Grounded on this, researchers developed a DNA vector-based technique which was expected to fold back and form a 19-nt stem-loop structure with a region homologous to the conserved sequences of 7 serotypes FMDV genome. The results indicated that a sequence-specific RNAi effect can be activated in cells by siRNAs derived from self-complementary hairpin-generating plasmids (Jacque *et al*., 2002).

The L protein and 3D protein would be good targets in the RNAi strategy to inhibit replication of FMDV. The L region consists of two overlapping proteins (Saiz *et al*., 2002), both of which catalyze their own cleavage from the rest of the polyprotein and also the cleavage of eIF-4G, leading to the shut off of host cells protein synthesis (Domingo and Escarmis, 2003). On the other hand, protein 3D is the viral RNA-dependent RNA polymerase which plays an essential role in viral replication. Due to its significance in viral replication, the *3D* gene is highly conserved, especially within the functional regions (Chen *et al*., 2003). Indeed, researchers demonstrated in the present study that shRNAs expressed by plasmids targeting to the selected conserved regions of *L* and *3D* gene of FMDV, effectively inhibited replication of FMDV. Researchers adopted several strategies to evaluate the antiviral effect of the designed shRNAs *in vitro* to begin with researchers monitored the fluorescence in both shRNA-expressing plasmids transfected cells and non-transfected cells to judge whether shRNAs could suppress the expression of fusion green fluorescent protein or not. Coherence to the expect, the frequency of GFP-expressing cell was obviously decreased when transfected with siRNAs whereas no fluorescence was seen in blank control cells and the control constructs (pEGFP-C1) gave significant expression of EGFP. Hence, the shRNAs could be elicited to inhibit the expression of FMDV *L* and *3D* gene in BHK-21 cells in a specific manner.

Since, shRNA-L could mediate more effective RNAi than that of shRNA-3D, estimated from fluorescence microscopic analyses, researchers secondly deal with the issue about the appropriate dose of shRNAs used for transfection to reach an ideal RNAi effect via increasing the concentration of pShuttle-shRNA-L. Microscope observation revealed that enhancing the siRNA-L quantity to 0.8 μg per well observably advanced the specific silencing efficacy and led to an almost complete loss of CPEs in BHK-21 cells. Consistent with the CPE results, virus titration revealed that 0.8 μg pShuttle-shRNA-L transfected cells showed $10^{2.25}$ $TCID_{50}$ at 24 h p.i. whereas, 0, 0.1, 0.2 and 0.4 μg shRNA transfected cells meanwhile showed virus titer of $10^{5.25}$, $10^{4.74}$, $10^{4.25}$, $10^{3.37}$ in supernatants, respectively. Notwithstanding, the mechanism is unclear (Scherr *et al*., 2005), the result presented evidence to display that the antivial effect was dose-independent.

Finally, researchers attempted to find out that whether virus replication in cells transfected with a mixture of different siRNAs could give the most significant effect (Kahana *et al*., 2004) by treating BHK-21 cells with a mixture of two different siRNA-expressing plasmids. To the surprise, quantitative RT-PCR results did not account for any synergistic effect of inhibiting FMDV replication. One reason may lie in the equal efficacy of the two shRNAs in inhibiting the replication of FMDV which was reflected by both viral titer detection and Q-RT-PCR investigation. Secondly, applying a mixture of different plasmids must cut down the dose of each shRNA and yet lack the consideration that the competence of these siRNAs may be discriminating. At present although, there is no satisfactory treatment for FMD, RNAi strategy might be considered as a feasible approach for controlling FMD outbreak. However, a number of important issues should be addressed. Recently, some viruses have been found to develop variants capable of escaping from the RNAi

effect (Boden *et al*., 2003; Das *et al*., 2004; Wu *et al*., 2005; Konishi *et al*., 2006). As for other RNA viruses, FMDV RNA genome is error-prone because of the lack of proofreading activity of the viral polymerase in the replication process (Biswas *et al*., 2006). Therefore, using expression systems delivering multiple siRNAs targeted to different conserved sequences simultaneously would be recommended.

CONCLUSION

This study demonstrated that pretreatment BHK-21 cells with vector-based, double-stranded siRNAs corresponding to the L and 3D non-structural protein coding region of FMDV genome could markedly lighten the CPE of BHK-21 cells, reduce the titration of virus progeny and promote clearance of the virus from most of the infected cells. Although, a number of important issues should be addressed before therapeutic application, researchers believe that RNAi should be an alternative strategy in the control of FMDV in an outbreak.

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