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# Inhibition of Foot and Mouth Disease Virus Replication *in vitro* and *in vivo* by Dual Short Hairpin RNA-Mediated RNA Interference

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**Abstract:** Foot and Mouth Disease Virus (FMDV) is one of the most important pathogen to the cattle industry often resulting in severe economic losses. Researchers have reported previously a therapeutic application of plasmid-based shRNA against FMDV but the high degree of sequence diversity between different FMDV serotypes may result in the appearance of escape mutants. In this study, a dual shRNA expression plasmid which can simultaneously express two different shRNA molecules was established and showed stronger inhibitory effects on virus replication than the mixture of two shRNAs. Moreover, the antiviral activity induced by the dual shRNA expression system was aslo evident on other FMDV serotypes. Therefore, the dual shRNA system targeting two conserved regions of virus genome provides a more powerful strategy for inhibiting FMDV replication in a cross-resistance manner and implicates a potential application in the treatment of high genetic variability of FMDV.

**Key words:** FMDV, RNA interference, dual shRNAs, plasmid, genome, serotypes

## INTRODUCTION

Foot and Mouth Disease (FMD) is an acute and highly contagious disease requiring expensive treatment occurring in cloven-hoofed animals (Pereira, 1981). The etiological agent of FMD is Foot and Mouth Disease Virus (FMDV) which belongs to the genus *Aphthovirus* of the family Picornaviridae. The spreading capacity of the virus and its ability to change its antigenic identity make it a serious threat to the beef and dairy industries in many countries (Bachrach, 1968; Howlader *et al*., 2004). Owing to the absence of reciprocal protection among 7 FMDV serotypes, it is difficult to control FMD through vaccination and impossible to eliminate FMD by conservative natural breeding (Knowles and Samuel, 2003). A recent occurrence of a large epidemiogenesis has made the development of emergency antiviral strategies essential for preventing outbreaks of FMD.

RNA interference (RNAi) is a process of sequence-specific, post-transcriptional gene silencing in animals and plants which can be induced by 21-23 nucleotide siRNA targeting corresponding mRNA (Fire, 1999; Bosher and Labouesse, 2000; Mittal *et al*., 2011). Specific gene silencing can be induced in cells by using synthetic siRNA, plasmid or virus-mediated shRNA. Inhibition of virus replication has been reported for a number of viruses like HBV (Kayhan *et al*., 2007), HIV (Coburn and Cullen, 2002), HCV (Kapadia *et al*., 2003), FMDV (Chen *et al*., 2004; Lv *et al*., 2009). The use of siRNA as an antiviral agent could lead to a selective pressure on the siRNA target sequences that might result in the appearance of escape mutants. Some alternative strategies for preventing escape mutants is to design multiple-siRNA expression system that focus on the conserved region of the viral genome. In this study, researchers constructed a dual shRNA expression plasmid system that can simultaneously produce two shRNAs targeting two different conserved region of FMDV genome and can cross-inhibit FMDV replication in BHK cells and suckling mice.

## MATERIALS AND METHODS

**Cells and viruses:** Baby Hamster Kidney (BHK) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Cultures were incubated at 37°C with 5% $CO_2$. FMDV serotypes O, A and Asia I were used for viral challenge.

**Design of shRNAs and plasmids:** Researchers reported previously that two different siRNA sequences (2B1 and 3D) could be used to effectively inhibit FMDV replication (Wang *et al*., 2010). The selected Target sequence (FMDV-2: GCT ACA GAT CAC CAT ACCT, FMDV-3: GCC AGA TGC AGA GGG ACAT) had 100% identity when compared with all the FMDV sequences deposited in GenBank, regardless of their serotype.

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Oligonucleotides of inverted repeat FMDV target sequence were annealed and cloned into the pGenesil-1 (Wuhan, Genesil Biotechnology Co., Ltd) to generate shRNA expression vectors pU6-sh2 and pU6-sh3. The sh3 expression cassette (including U6 promoter and sh3 sequence and termination signal) was amplified using primers U6-F (5'-TTG TCC AAA CTC ATC AAT GTA-3') and U6-R (5'-ACT TGA TTA GGG TGA TGG TTC-3') and cloned into SalI and EcoR I site of pU6-sh2 to generate a dual shRNA expression plasmid (pU6-sh3-U6-sh2) which simultaneously expressed two shRNAs.

**Cells, transfection and virus propagation:** BHK were seeded in 24 well plate (Costar) the day before transfection and cultured in fresh DMEM (10% FBS) without antibiotics to achieve 90% confluency. BHK were transfected with 2.5 μg plasmid DNA using Lipofectamine 2000 (Invitrogen). After 12 h transfection, the transfection complex was removed and the cells were washed twice with DMEM. The transfected cells in per well plates were then infected with 100 TCID50 of FMDV. 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 2% fetal bovine serum. After 48 h, cell supernatants were collected and the virus titers were determined three times on BHK cells. Statistical analysis was performed with the Microsoft Excel program (Microsoft, Redmond, WA).

**Quantitative real-time RT-PCR:** Viral RNA was isolated at 48 h after FMDV serotypes O infection using Trizol (Invitrogen) according to the manufacturer's instructions. From purified RNA, cDNA was synthesized using random hexamer primers and was quantified by spectrophotometer at 260 nm. Real-time PCR (Stratagene MX3000P) was carried out using SYBR Green (TaKaRa Biotech, Dalian) following the manufacturer's protocol. The following primers were used for FMDV amplification (FMDV-F 5'-CAAAAGATGGTCATGGGC-3' and FMDV-R 5'-CAACAGATGGCTACTGTCTTCCC-3'). Relative mRNA levels of target genes are presented as the ratio of the target gene product quantity to 1 ng beta-actin. Statistical analysis was performed with the Microsoft Excel Program (Microsoft, Redmond, WA).

**Viral challenge assay in suckling mice:** Kunming white mice were used for the detection the anti-FMDV activity of the shRNAs. The suckling mice were subcutaneously injected in the neck with 50-100 ug of shRNA plasmids dissolved in 100 uL of saline. After 6 h, the suckling mice were challenged with 20 LD50 of the FMDV serotypes O, A and Asia I per 0.1 mL by subcutaneous injection in the neck near the site that received the injected DNA and were then observed for 5-6 days post-challenge.

## RESULTS AND DISCUSSION

**Inhibition of FMDV replication in BHK cells by plasimid-based shRNA:** To test whether the dual shRNA expression plasmid or mixture of two shRNAs could enhance antiviral efficiency of RNAi, researchers compared antivirus activity against FMDV serotype O among pU6-sh3-U6-sh2, the mixture of pU6-sh3 and pU6-sh2 (pU6-sh3+pU6-sh2) and pU6-sh2. It is found that pU6-sh3-U6-sh2 induced a stronger antiviral effect than did pU6-sh3+pU6-sh2 ($p = 0.0437$) (Fig. 1a). The same results were obtained by the measurements of virus titers. pU6-sh3-U6-sh2 resulted in a reduction of approximately 100 fold in virus titer but the mixture of pU6-sh3+pU6-sh2 showed a decreased effect and there was no significant decrease of virus titers in pU6-shScr control (Fig. 1b).

**Dual shRNA expression plasmid inhibit replication of multiple FMDV serotypes in BHK cells:** One of concerns for shRNA antivirus is the antigenic variability of virus

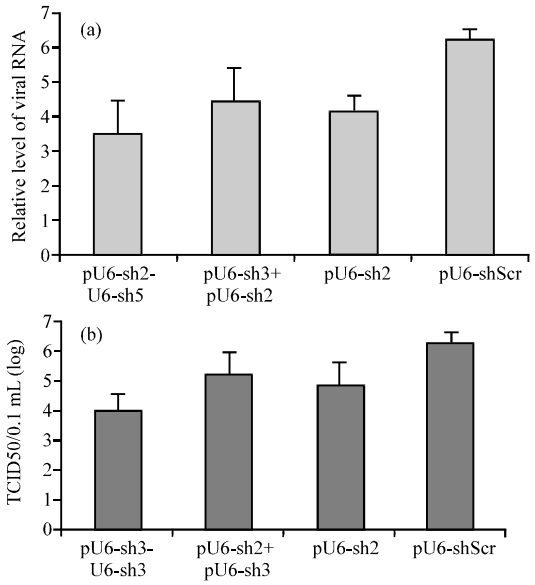


Fig. 1: Enhanced antiviral effect of dual shRNA expression plasmid in BHK cells. BHK cells were transfected with dual shRNA expression plasmid (pU6-sh3-U6-sh2), the mixture of two shRNA plasmid (pU6-sh2+pU6-sh3) or single shRNA expression plasmid (pU6-sh2) and then incubated for 1 h with FMDV serotype O; a) After 48 h post-infection, total RNAs were extracted from the supernatant and the relative levels of FMDV RNA were determined by real-time RT-PCR and b) Viral titers in the supernatants were determined 48 h pi by TCID50 assay. A scrambled shRNA plasmid (pU6-shScr) was used as a negative control. Error bars indicate Standard Deviation (SD)

and escape mutant. To test whether the pU6-sh3-U6-sh2 could inhibit other FMDV serotypes, the transfected BHK cells were infected with FMDV serotypes O, A and Asia I. After 48 h viral challenge, lysed cell supernatants were analyzed by TCID50. As shown in Fig. 2, the replication of O, A and Asia1 types was significantly inhibited. The TCID50 of the FMDV serotypes O, A and Asia I in supernatants of cells transfected with pU6-sh3-U6-sh2 and pU6-sh2 was lower than that in the control cells. In addition, differences in the degree of inhibition among FMDV serotypes O, A and Asia I were not significant ($p>0.05$).

**Dual shRNA expression plasmid induced cross-resistance to heterologous FMDV infection in suckling mice:** To test anti-FMDV potential of the dual shRNA expression plasmid *in vivo*, researchers challenged suckling mice, pretreated by subcutaneous injection of shRNA plasmids with FMDV serotype O. All pU6-shScr control treated mice died within 60 h after the viral challenge. The pU6-sh3-U6-sh2 and pU6-sh2 plasmid inhibited viral replication of FMDV in suckling mice (Fig. 3a). Treatment with pU6-sh3-U6-sh2 improved the survival rate of mice compared with pU6-sh2. The 6 of 40 mice pretreated with pU6-sh2 and 22 of 40 mice pretreated with pU6-sh3-U6-sh2 survived a viral challenge of 20 LD50 for 6 days of observation. The difference between the survival rate of mice treated with pU6-sh3-U6-sh2 and pU6-shScr control was statistically significant ($p<0.00001$). To demonstrate the antiviral effects against other serotypes of FMDV for the dual shRNA expression plasmid, researchers challenged suckling mice with FMDV serotypes A and Asia I after injection the pU6-sh3-U6-sh2, pU6-sh2 or pU6-shScr. In FMDV serotype A challenge experiments, 4 of 40 mice treated with pU6-sh2 were protected (Fig. 3b). However, 19 of 39 mice pretreated with pU6-sh3-U6-sh2 survived a viral challenge of 20 LD50 for 6 days of observation (Fig. 3b). In the case of challenge with 20 LD50 Asia I, 4 of 39 mice treated with pU6-sh2 and 15 of 40 mice pretreated with pU6-sh3-U6-sh2 survived (Fig. 3c). For FMDV serotypes A and Asia I challenge, a significant difference in mouse survival rate was also seen between mice treated with pU6-sh3-U6-sh2 and pU6-shScr controls ($p<0.00001$).

The application of siRNA to FMDV is a promising tool for virus inhibition and can be applied for both preventive and therapeutic approaches (Kim *et al*., 2008). This suggests that siRNA can be used as a potential alternative strategy for FMDV control. However, long-term silencing of viral protein expression by siRNA has been reported to lead to the emergence of viruses

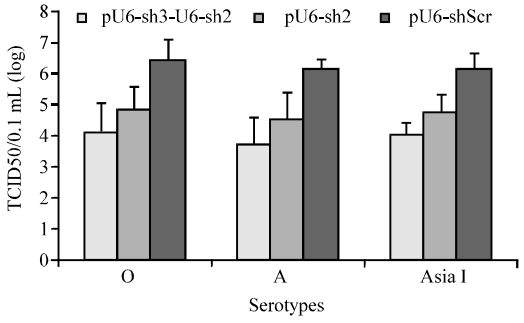


Fig. 2: Dual shRNA expression plasmid inhibits replication of multiple FMDV serotypes. BHK cells were transfected with pU6-sh3-U6-sh2, pU6-sh2 or control plasmid (pU6-shScr) and then infected with FMDV serotypes O, A and Asia I, respectively. Virus titers were determined by TCID50 assay after 48 h infection. A scrambled shRNA plasmid (pU6-shScr) was used as a negative control. Error bars indicate Standard Deviation (SD)

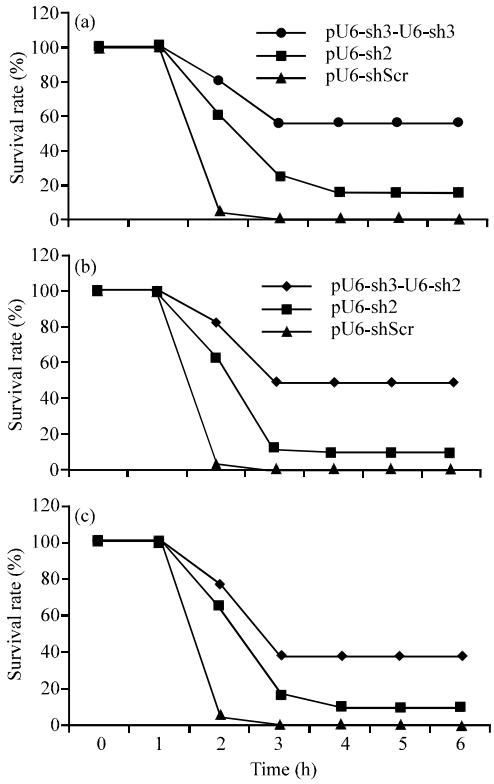


Fig. 3: Cross-resistance effects in suckling mice by dual shRNA expression plasmid. Suckling mice were challenged by subcutaneous injection in the neck with 20 LD50 of; a) FMDV serotypes O; b) A; c) Asia I after 6 h of treatment with 100 μg pU6-sh3-U6-sh2, pU6-sh2 or control plasmid (pU6-shScr). All animals were observed for 6 days after challenge

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. 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; Geisbert *et al*., 2006) may be an approach to resolve this issue. The strategies to address these potential problems are to generate dual shRNA molecules that can target two different conserved region of the viral genome (Gitlin *et al*., 2005).

In this study, researchers constructed a dual shRNA expression plasmid pU6-sh3-U6-sh2. To test whether this system can be used to enhance the efficiency of inhibiting FMDV replication, researchers compared anti-FMDV effects between the dual shRNA and the mixture of single shRNA. The dual shRNAs driven by two U6 significantly improve antiviral effects but the mixture of single shRNA show no improvement of antiviral effects. In fact, the antiviral effect decreased a little (Fig. 1). Chen and Mahato (2008) reported a similar result that one plasmid expressing three shRNAs against HBV was much stronger than the application of several pooled shRNAs. This result showed that the strategy of expressing siRNAs simultaneously in one plasmid is more effective than the strategy of mixing siRNAs. In addition, we also observed significant antiviral activity against FMDV serotypes A and Asia I when using the dual shRNA system to expressed two shRNAs in BHK cells and in suckling mice. These results indicate that the dual shRNAs targeted conserved regions of FMDV genome not only conferred a clear reduction in homologous FMDV but also permitted the inhibition of other FMDV serotypes.

## CONCLUSION

This study shows that plasmid-based shRNAs targeting conserved regions of FMDV genome were efficient for inhibition of FMDV replication. Co-expression of dual shRNAs in one plasmid can improve antiviral efficiency of siRNA and permitted the induction of cross-resistance to FMDV infection. Thus, this approach would be beneficial especially to those viruses with high genetic variability like FMDV and BVDV. In addition, the dual shRNA expression may be used to deal with multi-virus coinfection by expressing multiple shRNAs targeting different virus.

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## REFERENCES

Bachrach, H.L., 1968. Foot-and-mouth disease. Annu. Rev. Microbiol., 22: 201-224.

Bosher, J.M. and M. Labouesse, 2000. RNA interference: genetic wand and genetic watchdog. Nat. Cell. Biol., 2: 31-36.

Chang, L.J., X. Liu and J. He, 2005. Lentiviral siRNAs targeting multiple highly conserved RNA sequences of human immunodeficiency virus type 1. Gene. Ther., 12: 1133-1144.

Chen, W., W. Yan, Q. Du, L. Fei and M. Liu *et al*., 2004. RNA interference targeting VP1 inhibits foot-and-mouth disease virus replication in BHK-21 cells and suckling mice. J. Virol., 78: 6900-6907.

Chen, Y. and R.I. Mahato, 2008. siRNA pool targeting different sites of human hepatitis B surface antigen efficiently inhibits HBV infection. J. Drug. Target., 16: 140-148.

Coburn, G.A. and B.R. Cullen, 2002. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J. Virol., 76: 9225-9231.

Das, A.T., T.R. Brummelkamp, E.M. Westerhout, M. Vink, M. Madiredjo, R. Bernards and B. Berkhout, 2004. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J. Virol., 78: 2601-2605.

Dave, R.S. and R.J. Pomerantz, 2004. Antiviral effects of human immunodeficiency virus type 1-specific small interfering RNAs against targets conserved in select neurotropic viral strains. J. Virol., 78: 13687-13696.

Fire, A., 1999. RNA-triggered gene silencing. Trends. Genet., 15: 358-363.

Geisbert, T.W., L.E. Hensley, E. Kagan, E.Z. Yu and J.B. Geisbert *et al*., 2006. Postexposure protection of guinea pigs against a lethal Ebola virus challenge is conferred by RNA interference. J. Infect. Dis., 193: 1650-1657.

Gitlin, L., J.K. Stone and R. Andino, 2005. Poliovirus escape from RNA interference: Short interfering RNA-target recognition and implications for therapeutic approaches. J. Virol., 79: 1027-1035.

Howlader, M.M.R., A.T.M. Mahbub-E-Elahi, S. Habib, M.J.U. Bhuyian, M.A.B. Siddique, M.A. Hai and M.G. Hossain, 2004. Foot and mouth disease in baghabari milk shed area and it`s economic loss in Bangladesh. J. Biological Sci., 4: 581-583.

Kapadia, S.B., A. Brideau-Andersen and F.V. Chisari, 2003. Interference of hepatitis C virus RNA replication by short interfering RNAs. P. Natl. Acad. Sci. USA., 100: 2014-2018.

Kayhan, H., E. Karatayli, A.R. Turkyilmaz, F. Sahin, C. Yurdaydin and A.M. Bozdayi, 2007. Inhibition of hepatitis B virus replication by shRNAs in stably HBV expressed HEPG2 2.2.15 cell lines. Arch. Virol., 152: 871-879.

Kim, S.M., K.N. Lee, J.Y. Park, Y.J. Ko and Y.S. Joo *et al*., 2008. Therapeutic application of RNA interference against foot-and-mouth disease virus *in vitro* and *in vivo*. Antiviral. Res., 80: 178-184.

Knowles, N.J. and A.R. Samuel, 2003. Molecular epidemiology of foot-and-mouth disease virus. Virus. Res., 91: 65-80.

Lv, K., Y. Guo, Y. Zhang, K. Wang, K. Li, Y. Zhu and S. Sun, 2009. Transient inhibition of foot-and-mouth disease virus replication by siRNAs silencing VP1 protein coding region. Res. Vet. Sci., 86: 443-452.

Mittal, P., R. Yadav, R. Devi, A. Tiwari, S.P. Upadhye and S.S. Gosal, 2011. Wondrous RNAi-gene silencing. Biotechnology, 10: 41-50.

Pereira, H.G., 1981. Foot and Mouth Disease Animals. Academic Press, New York, pp: 333-363.

Wang, P.Y., J.J. Jiang, N. Li, J.L. Sheng and Y. Ren *et al*., 2010. Transgenic mouse model integrating siRNA targeting the foot and mouth disease virus. Antivir. Res., 87: 265-268.