Antiviral Research 87 (2010) 307–317

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Multiple shRNAs driven by U6 and CMV promoter enhances efficiency of antiviral effects against foot-and-mouth disease virus

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ARTICLE INFO

Article history:
Received 3 June 2009
Received in revised form 10 May 2010
Accepted 8 June 2010

Keywords:
Foot-and-mouth disease virus
Small interference RNA
Adenovirus
Multiple shRNAs
Promoters

ABSTRACT

Foot-and-mouth disease (FMD) is an economically significant animal disease because of the speed of its transmission. The current vaccine for FMD provides no protection until 7 days post-vaccination, thus reducing its effectiveness in the case of an outbreak. Small interfering RNA (siRNA) is a promising antiviral approach because it can induce a protective response much more rapidly. This study is the first report to apply multiple short hairpin RNA (shRNA) expression systems to inhibit foot-and-mouth disease virus (FMDV) replication. Three different shRNAs, one targeting 2B region and two targeting 3C region, were driven by three RNA Polymerase III (Pol III) promoters, U6 or a combination of two U6 promoters and one RNA Polymerase II (Pol II) promoter, CMV. The adenoviruses simultaneously expressing three different shRNAs in a single construct had significantly enhanced antiviral effects compared with those expressing only a single shRNA, those expressing double shRNAs or a mixture of adenoviruses expressing a single shRNA and the adenovirus expressing double shRNAs, both *in vitro* and *in vivo*. The adenoviruses had broad antiviral effects against seven serotypes of FMDV, including O, A, Asia1, C, SAT1, SAT2, and SAT3 *in vitro*, but differed in their efficacy. The adenovirus expressing multiple shRNAs driven by three U6 promoters had strong antiviral effects in suckling mice challenged with O, A, and Asia1 serotype of FMDV.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease that affects cloven-hoofed animals, such as cattle, swine and sheep (Pereira, 1981). The ability of foot-and-mouth disease virus (FMDV) to spread rapidly in susceptible animals makes FMD a disease that is serious enough to be listed by the World Organization for Animal Health (OIE). FMD is one of the most significant political and economic animal diseases in the world. FMDV belongs to the genus *Aphthovirus* in the family *Picornaviridae* (Bachrach, 1968). The virus genome is 8.5 kb positive-sense single stranded RNA containing one open reading frame. There are seven serotypes: A, O, Asia1, C, SAT1, SAT2, and SAT3, but numerous subtypes have evolved within each serotype (Knowles and Samuel, 2003).

The phenomenon of RNA interference (RNAi) is mediated by short hairpin RNA (shRNA), and causes gene silencing in a sequence-specific manner. Inhibition of virus replication by RNA interference has been primarily reported for a number of human disease viruses, including poliovirus, human immunodeficiency virus type 1 (HIV-1), and hepatitis (Gitlin et al., 2005; Hamasaki et al., 2003; Jacque et al., 2002; Lee and Rossi, 2004; Saulnier et al., 2006). Recently, researchers have reported inhibition of FMDV replication using plasmid or adenovirus expressing RNA interference (Chen et al., 2006, 2004; Kahana et al., 2004). We have reported a therapeutic application of adenovirus expressing shRNA against FMDV (Kim et al., 2008).

Small interfering RNA (siRNA) represents one potential approach for inhibiting FMDV replication. However, a single-point mutation of the target sequence has been shown to be sufficient to completely abolish the silencing efficiency of siRNAs in several cases (Pusch et al., 2003; Sabariegos et al., 2006). The siRNA method has limitations in protecting against FMDV that have a high mutation rates or several serotypes. In addition, viruses have the ability to generate a spectrum of mutants resistant to siRNAs, as

Abbreviations: FMD, foot-and-mouth disease; FMDV, foot-and-mouth disease virus; GFP, green fluorescent protein; siRNA, small interference RNA; shRNA, short hairpin RNA; RNAi, RNA interference; miRNA, microRNA; mRNA, messenger RNAs; Pol II, RNA polymerase II; Pol III, RNA Polymerase III; $TCID_{50}$, 50% tissue culture infective dose; RT-PCR, reverse transcriptase polymerase chain reaction; p.i., post-infection; IFN, interferon; ISGs, interferon α/β stimulate genes; PKR, double stranded RNA-dependent protein kinase (PKR); OAS, 2′–5′ oligoadenylate synthetase; GAPDH, glyceraldehyde 3-phophate dehydrogenase; DPC, days post-challenge; IP, intraperitoneal; LD_{50}, 50% lethal dose.

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doi:10.1016/j.antiviral.2010.06.004

has been discovered for poliovirus (Gitlin et al., 2005). An alternative approach for preventing this emergence of escape mutants has been the simultaneous use of multiple shRNAs (Gitlin et al., 2005; ter Brake et al., 2006). Researchers have demonstrated that the hepatitis B virus (HBV) could be effectively inhibited using dual siRNAs driven by the RNA Polymerase III (Pol III) promoter, U6 (Wu et al., 2005). In another study, a plasmid encoding three short hairpin RNAs (shRNAs) driven by a single U6 promoter against HBV was more effective than a plasmid encoding single shRNA in cells (Chen and Mahato, 2008).

In previous study on multiple shRNAs against HIV-1, researchers used combination of U6, H1, and 75K (Pol III) pomoter and U1 (Pol II) promoter to avoid recombination of multiple promoters in lentiviral system (ter Brake et al., 2008). They demonstrated that multiple shRNAs driven by combination of promoters was more effective than those driven by three H1 promoters. However, they did not test cell toxicities. In another study using multiple shRNAs against HIV-1, reserchers used a lentiviral system and showed that a combination of pol II and pol III promoters to express two siRNAs increased the afficacy without toxicity (Lo et al., 2007). They showed that a single shRNA-driven U6 promoter had high efficacy but affected cell viability before 7 days after tranduction in cells. They suggested that the results were due to shRNA overexpression driven by U6 promoter that competed with endogeneous microRNAs (miRNAs).

This study is the first report to apply multiple shRNA expression systems to inhibit foot-and-mouth disease virus (FMDV) replication. To enhance antiviral efficiency against FMDV, we developed an adenovirus system that expresses three shRNAs driven by three U6 promoters. The U6 promoter is well characterized, a strong pol III promoter, and more efficient than the H1 promoter (Lo et al., 2007; Mäkinen et al., 2006). To counteract any potential toxicity induced by shRNA overexpression driven by Pol III promoters (Grimm et al., 2006), we also developed an adenovirus that expressed three shRNAs that were driven by a combination of two Pol III (U6) promoters and one Pol II (CMV) promoter. We compared the recombinant adenovirus expressing three shRNAs under control of three U6 promoters with that expressing three shRNAs under control of two U6 promoters and one CMV promoter in antiviral efficiency and cytotoxicity. we showed that two adenoviruses simultaneously expressing three different shRNAs had high efficiency and inhibited against O, A, Asia1, C, SAT1, SAT2 and SAT3 serotypes of FMDV replication *in vitro*. In addition, we showed that the recombinant adenovirus expressing three shRNAS under control of three U6 promoters has strong antiviral effects in animals although expression of three siRNAs from U6 promoters led to toxicity and increased the level of mRNAs related to type I interferon in IBRS-2 cells.

2. Materials and methods

2.1. Cells, animals, viruses and virus titration

Human embryonic kidney cells, including human adenovirus type 5 E1 DNA (293 A cells), and swine kidney cells (IBRS-2), were propagated in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS; pH 7.4) at 37 °C with 5% CO_2. CD-1 (ICR) suckling mice, 6 days old and weighing 4–5 g, were purchased from Orient Co. Ltd (Republic of Korea). Animals were treated in accordance with the ethical guidelines of the animal welfare committee of the National Veterinary Research and Quarantine Service (NVRQS). Strains of FMDV, O/SKR/2002, O1 Manisa, Asia1/MOG/05, A22/IRQ 24/64, C3/Resende, SAT1/BOT 1/68, SAT2/ZIM 5/81 and SAT3/ZIM 4/81 were used for viral challenges.

Virus titers were determined in IBRS-2 cells for FMDV and 293A cells for adenovirus. The 50% tissue culture infective dose ($TCID_{50}$) was calculated using the formula of Reed and Muench (Reed and Muench, 1938).

2.2. Design and construction of plasmid

We have reported previously that two different siRNA sequences (2B and 3C1) could be used to inhibit FMDV replication (Kim et al., 2008). These siRNA sequences were used in this study and we designed one more siRNA (3C2). The siRNAs were developed in reference to FMDV O/SKR/2000 (GenBank accession number AJ539139) and O/SKR/2002 (GenBank accession numbers AY312589 and AY312588) strain sequences using Blast and Turbo si-Designer Program (Bioneer Corporation, Republic of Korea). The target sequences of the siRNAs are summarized in Table 1. Oligonucleotides of inverted repeat FMDV target sequence were annealed and cloned into the pENTR™/U6 entry vector (Invitrogen, The Netherlands) under control of the U6 promoter, or pSilencer™ adeno1.0 (Ambion) under control of the modified CMV promoter. We amplified the promoter (U6 or modified CMV), shRNA sequence and termination signal (Pol III or Pol II terminator) using primers (Table 2) and one PCR product were cloned into Sal I and Kpn I site to generate dual shRNA expression cassettes and other PCR products were cloned into Kpn I site of dual shRNA expression cassettes to generate three different shRNA cassettes (Fig. 1). Negative control shuttle vector (Ambion) with scrambled siRNA sequence which lacks homology to the mouse, human, and rat was used as a control in this study.

2.3. Recombinant adenovirus production

Recombinant human adenovirus produced by the Manufacturer's instructions. Briefly, to generate recombinant adenovirus expressing single shRNA under control of the U6 promoter and expressing two and three shRNAs, we performed an LR recombination reaction using pENTR™/U6 entry construct and pAd/BLOCK-iT-DEST vector (Invitrogen), and 293A cells were transfected with the adenovirus constructs digested with *Pac* I using Lipofectamine 2000 (Invitrogen). To generate recombinant adenovirus expressing single shRNA under control of the CMV promoter, 293A cells were cotransfected with Pac I digested Adenoviral LacZ back bone and/or pSilencer™ adeno1.0 shuttle vector (Ambion) using calcium chloride. When approximately 80% CPE was observed, the adenovirus was harvested and amplified. Adenovirus stock of 10^8 to 10^9 $TCID_{50}$/ml was used for the experiments.

2.4. Ad-shRNA inoculation and viral challenge in IBRS-2 cells

IBRS-2 cells of 3.5×10^4 were plated in each well of 96-well plates. On the following day, plated cells were 90% confluent at the time of infection. Cells were inoculated with recombinant adenovirus at 3×10^6 $TCID_{50}$ or 1×10^7 $TCID_{50}$. After a 12 h absorption, the adenovirus suspension was removed and cells were washed twice with D-MEM. IBRS-2 cells were immediately infected with 100 $TCID_{50}$ of FMDV (12 h post-inoculation of adenoviruses). After a 1 h adsorption period at 37 °C, the inoculums were removed and cells were washed twice with D-MEM. The culture medium of 200 μl with 2% FBS was added and cells were incubated at 37 °C. Supernatant was collected at 24 h and 48 h post-FMDV infection to demonstrate the antiviral effects of adenovirus expressing single and dual shRNA, and at 54 h post-infection to demonstrate the antiviral effects of adenovirus expressing three shRNAs. The supernatant was used in kinetic analysis of FMDV.

Table 1
Target sequences of shRNAs used in this study.

Name	Target sequence (nucleotide position of O/SKR/2002)	Oligonucleotide sequence
Ad-U63C1	5′-GAGTGTTTGAGTTTGAGAT-3′ (nt 6240–6258 in the 3C region)	5′-CACCGGAGTGTTTGAGTTTGAGATCGAAATCTCAAACTCAAACACTC-3′(Top strand) 5′-AAAAGAGTGTTTGAGTTTGAGATTTCG ATCTCAAACTCAAACACTCC-3′ (Bottom strand)
Ad-U62B	5′-GCAGGAGGACATGTCAACA-3′ (nt 4012–4030 in the 2B region)	5′-CACCGGCAGGAGGACATGTCAACACGAATGTTGACATGTCCTCCTGC-3′(Top strand) 5′-AAAAGCAGGAGGACATGTCAACATTCGATGTTGACATGTCCTCCTGCC-3′(Bottom strand)
Ad-U63C2	5′-GACAGTGACTACAGAGTGT-3′ (nt 6227-nt 6245 in the 3C region)	5′-CACCGGACAGTGACTACAGAGTGTCGAAACACTCTGTAGTCACTGTC-3′ (Top strand) 5′-AAAAGACAGTGACTACAGAGTGTTTCGACACTCTGTAGTCACTGTCC-3′(Bottom strand)
Ad-CMV3C2	5′-GACAGTGACTACAGAGTGT-3′ (nt 6227-nt 6245 in the 3C region)	5′-TCGAGGACAGTGACTACAGAGTGTTTCAAGAGAACACTCTGTAGTCACTGTCAGA-3′(Top strand) 5′-CTAGTCTGACAGTGACTACAGAGTGTTCTCTTGAAACACTCTGTAGTCACTGTCC-3′(Bottom strand)

Sense and antisense target sequences are underlined.

Table 2
Primers used in this study.

Name (restriction site of endonuclease)	Oligonucleotide sequence	Final product
U6 F(Sal I) U6 R(Kpn I)	5′-TTGTCGACAAGGTCGGGCAGGAAG-3′ 5′-TTGGTACCGTACAAGAAAGCTGGGTCT-3′	Ad-U62B-U63C1
U6 F(Kpn I) U6 R(Kpn I)	5′-TTGGTACCAAGGTCGGGCAGGAAG-3′ 5′-TTGGTACCGTACAAGAAAGCTGGGTCT-3′	Ad-U62B-U63C1-U63C2
CMV F(Kpn I) CMV R(KpnI)	5′-TTGGTACCTAGTTATTAATAGTAATC-3′ 5′-TTGGTACCCCGCGGGGACTAGAGTCG-3′	Ad-U62B-U63C1-CMV3C2

The restriction sites of endonuclease are underlined.

2.5. Analysis of FMDV replication in IBRS-2 cells

To assay the effects of adenovirus-mediated shRNA on FMDV RNA replication, quantitative real-time RT-PCR and virus titrations were carried out. Viral RNA was extracted by the BioRobot M48 workstation and MagAttract viral RNA M48 kit (Qiagen). Real-time RT-PCR was conducted using the one step Primescript RT-PCR kit (TAKARA, Otsu, Shiga, Japan). These procedures were conducted according to the manufacturer's instructions. Primers targeting 3D region were sense 5′-GGAACYGGGTTTTAYAAACCTGTRAT-3′ and antisense 5′-CCTCTCCTTTGCACGCCGTGGGA-3′. The probe was 5′-CCCADCGCAGGTAAAGYGATCTGTA-3′ and its 5′end was labeled with 6-FAM and the 3′end was labeled with TAMRA. Primers and Taqman probe were designed with Primer Express software

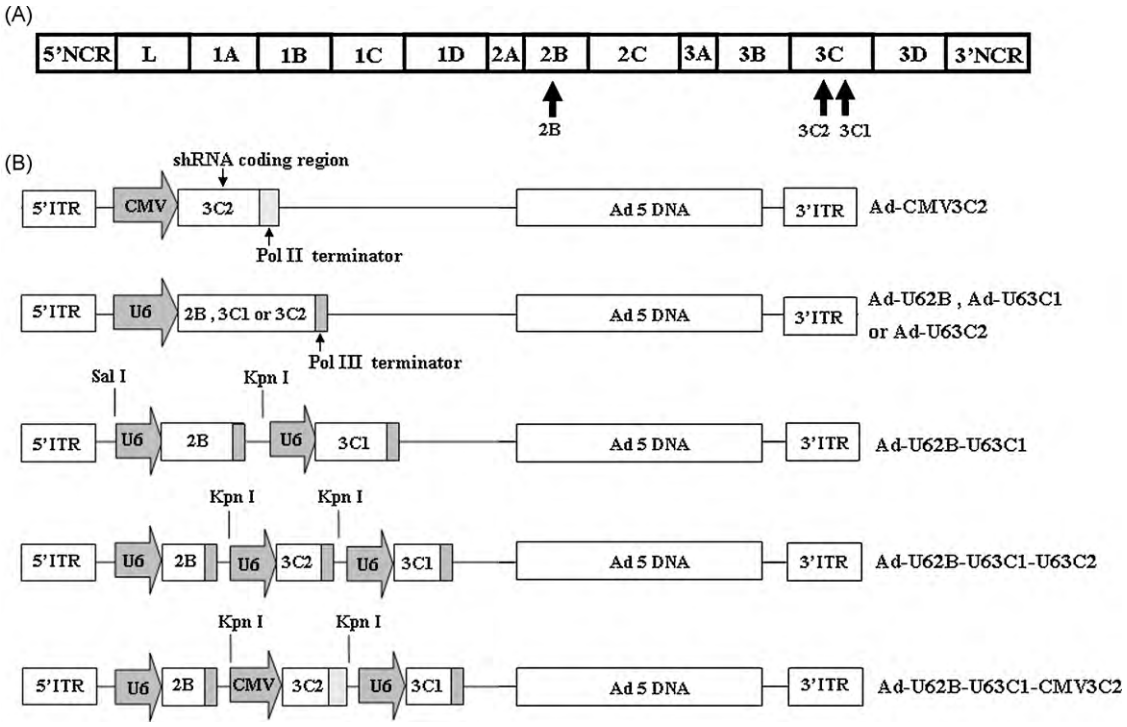

Fig. 1. Schematic representation of the generation of recombinant adenoviruses. (A) Diagram of the FMDV full genome. The black arrows indicate the target region of shRNA. (B) Schematic representation of recombinant adenovirus DNAs. Adenovirus DNAs expressing two or three shRNAs were cloned by restriction enzyme sites. Each adenovirus DNA contains promoter(s) (CMV or U6), shRNA coding region(s) and transcription terminator(s). A light grey box indicates Pol II terminator and a dark grey box indicates Pol III terminator.

(Applied Biosystems). Amplification and product detection were performed by the ABI 7500 Real-time PCR System (Applied Biosystems).

Virus titer ($TCID_{50}$) of the supernatant was determined in IBRS-2 cells. T-test was performed for statistical analysis by Graph Pad Instat software (Ver 3.05).

2.6. RT-PCR for the siRNA target region and sequencing

The nucleotide sequencing for six strains – O1 Manisa (GenBank accession number AY593823), Asia1/MOG/05 (GenBank accession number EF614458), A22/IRQ 24/64 (GenBank accession number AY593753), C3/Resende (GenBank accession number AY593807), SAT1/BOT 1/68 (GenBank accession number AY593845), and SAT2/ZIM 5/81 (GenBank accession number AF540910) – aimed to check minor mutations. Sequencing for the SAT3 ZIM 4/81 strain checked for minor mutation of 3C region (GenBank accession number AY884148) and sought to obtain unknown sequences of the 2B region. Viral RNAs of seven serotypes of FMDV (O1 Manisa, Asia1/MOG/05, A22/IRQ 24/64, C3/Resende, SAT1/BOT 1/68, SAT2/ZIM 5/81, and SAT3/ZIM 4/81) used in this study were extracted by BioRobot M48 workstation (Qiagen). Reverse transcription was performed using Superscript III (Invitrogen) and oligodT primer and cDNA was amplified using AccuPower High fidelity PCR-premix (Bioneer, Republic of Korea). PCR product was purified using Gel Extraction kit (Qiagen) and sequenced by 3130 Genetic analyzer (Applied Biosystems). The primers for amplifying and sequencing 2B region were: F12_3690, 5′-GACATGTCCTCCTGCATCTG-3′, and 2BR, 5′-TTGTACCAGGGYTTGGCYT-3′ and 3C1 and 3C2 regions were: 3B3F, 5′-CGCTTTGAAAGTGAAAGCTA-3′, 3C1R, 5′-CGCTCTTCCACATCTCTGGT-3′. Sequence alignment and editing were performed using CLUSTAL W (Thompson et al., 1994) and BioEdit program (Hall, 1999).

2.7. Silencing effects of the siRNA driven by U6 or modified CMV promoter

To demonstrate the silencing effects of the third siRNA driven by U6 or modified CMV promoter, 3C2 siRNA was replaced with Green fluorescent protein (GFP) siRNA (Darcan-Nicolaisen et al., 2009) in pENTR™/U6 entry plasmid expressing three siRNA, 2B, 3C1 and 3C2. The oligonucleotides of inverted repeat GFP target sequences were annealed, amplified and cloned into the plasmid expressing 2B and 3C1 siRNA, as described in the design and construction of plasmids (Materials and Methods). IBRS-2 cells, measuring 3.5×10^4, were plated in each well of the 96-well plates. On the following day, plated cells were 90% confluent at the time of transfection. Co-transfections of 4 μg of the plasmid expressing three siRNAs (2B, 3C1, and GFP siRNA) or expressing two siRNAs (2B and 3C1) and 0.5 μg of target plasmid, pIRES2-AcGFP (Clontech), was carried out with Lipofectamine 2000 (Invitrogen) as described by the Manufacturer. After 48 h, the cells were examined microscopically for GFP expression. Images were collected with an Olympus 1×70 inverted microscope and an Olympus U-TVO, $5 \times$ C-3 camera.

2.8. Cell viability test

Flow cytometry, Guava EasyCyte Plus system (Guava Technologies, Billerica, MA) was used to test cytotoxicity of recombinant adenoviruses expressing three shRNAs. IBRS-2 cells of 1×10^5 were plated in each well of 24-well plates and inoculated with 5×10^7 $TCID_{50}$ of adenovirus at the following day. After a 2-h adsorption at 37 °C, the inoculums were removed and cells were washed twice with D-MEM. The culture medium of 800 μl with 2% FBS was added and cells were incubated at 37 °C. Cells were collected at 48 h post-adenovirus infection and suspended with culture medium of 500 μl. Suspended cells were stained according to Manufacturer's instructions using Guava ViaCont Reagent (Guava Technologies) containing DNA-binding dyes. Briefly, cell suspension of 50 μl and reagent of 450 μl were gently mixed and incubated for 5 min. Stained cells were measured by Guava EasyCyte Plus system (Guava Technologies) and analyzed using CytoSoft™ software (Guava Technologies) to determine the proportion of viable cells.

2.9. Analysis for type I interferon mRNA

To analyze the level of type I interferon (IFN) mRNA and IFN α/β stimulated genes (ISGs) mRNA, a quantitative relative real-time RT-PCR was used to evaluate the mRNA levels of porcine interferon-alpha (IFN-α), porcine interferon-beta (IFN-β), double stranded RNA-dependent protein kinase (PKR), and 2′–5′ oligoadenylate synthetase (OAS). IBRS-2 cells of 3.5×10^5 were plated in each well of 12-well plates and inoculated with 8×10^7 $TCID_{50}$ of adenovirus at the following day. After a 2-h adsorption at 37 °C, the inoculums were removed and cells were washed twice with D-MEM. The culture medium of 800 μl with 2% FBS was added and cells were incubated at 37 °C. Cells were collected at 12 post-adenovirus infection and total RNAs were extracted using an RNeasy mini kit (Qiagen). Approximately 1ug of RNA was treated with DNase I (Sigma) and after heat inactivation of the enzyme, cDNA was synthesized using Superscript III (Invitrogen) and oligodT primer. Primers and Taqman probes described previously were purchased from Applied Biosystems (de los Santos et al., 2005). Porcine glyceraldehyde 3-phophate dehydrogenase (GAPDH) was used as an internal control, and cells treated with the adenovirus expressing porcine interferon-α (Ad-PoIFNα) were used a positive control.

2.10. Viral challenge in suckling mice

The animal experiment was approved by Animal Care and Use Committee of National Veterinary Research and Quarantine Service (NVRQS). To investigate the antiviral effects *in vivo*, 6-day-old suckling mice were used in this study. The dose of FMDV was determined in four 10-fold serial dilutions of the virus. The 50% lethal dose (LD_{50}) of FMDV used was estimated by the Reed-Muench method (Reed and Muench, 1938). The inoculation time and method followed those of the previous study (Kim et al., 2008). Briefly, suckling mice were inoculated by intraperitoneal (IP) injection with 6×10^7 $TCID_{50}$ (Fig. 6A and B) or 6×10^6 $TCID_{50}$ (Fig. 6C and D) of adenovirus. After 6 or 24 h, the suckling mice were challenged 125 LD_{50} of FMDV (O/SKR/2002, A22/IRQ 24/64 or Asia1/MOG/05) of 0.05 ml by IP injection. The animals were monitored for 7 days. The log-rank test was performed for statistical analysis by GraphPad Prism (Version 4.0).

3. Results

3.1. Inhibition of FMDV replication in IBRS-2 cells by adenovirus expressing single shRNA or dual shRNAs

The level of FMDV RNA and the viral titer were reduced in cells infected with adenovirus-mediated shRNAs (Fig. 2). The antiviral effects of Ad-U63C1 (called Ad-3C in our previous study) and Ad-U62B were proven, and their mixture was less effective than Ad-U63C1 had been *in vitro* in the previous study (Kim et al., 2008).

We produced Ad-U62B-U63C1, which was a recombinant adenovirus that simultaneously targeted the 3C1 and 2B regions (Fig. 1B). When we analyzed the level of viral RNA at 48 h post-FMDV infection, the higher inhibitory effects of the Ad-U62B-U63C1 inoculation group were statistically significant compared to

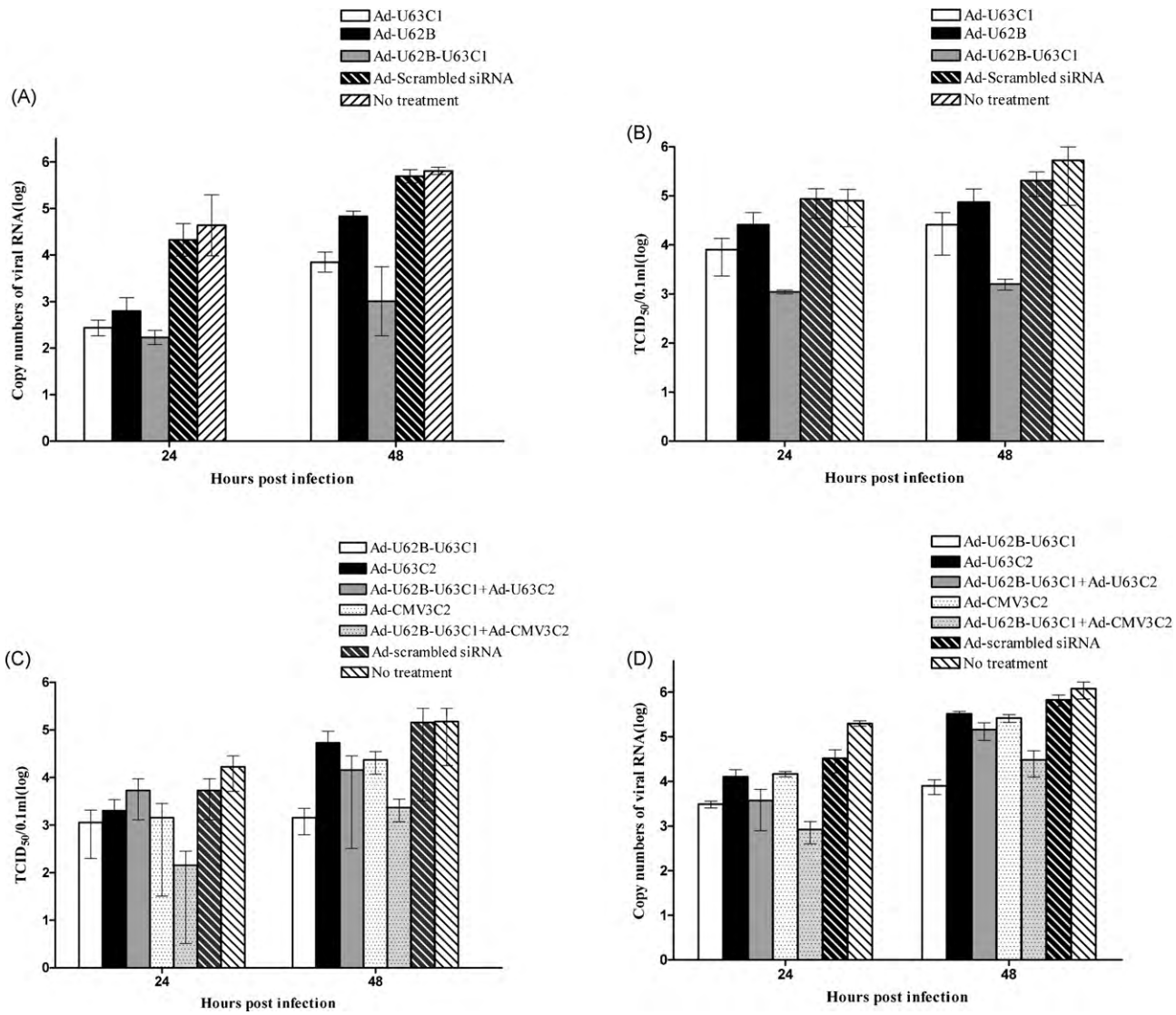

Fig. 2. Enhanced antiviral effect of adenovirus expressing dual shRNAs in IBRS-2 cells compared with adenovirus expressing single shRNA. Cells were inoculated with Ad-shRNA at 3×10^6 $TCID_{50}$. After 12 h, IBRS-2 cells were infected with 100 $TCID_{50}$ of FMDV O/SKR/2002. Samples of supernatant were taken at 24 and 48 h following FMDV infection. The amount of FMDV RNA was determined by quantitative real-time RT-PCR. Total RNA was extracted from the supernatant and used for quantitative real-time RT-PCR (A and C). The titer of FMDV was determined by $TCID_{50}$ in IBRS-2 cells (B and D). Ad-U62B-U63C1 represents an adenovirus that simultaneously expresses 2B and 3C1 shRNAs driven by U6 promoters, and Ad-U62B-U63C1 + Ad-U63C2 denotes an adenovirus mixture of Ad-U62B-U6-3C1 and Ad-U63C2. Recombinant adenovirus with scrambled siRNA sequence was used as a negative control. Error bars indicate standard deviation (SD).

the Ad-U62B and Ad-U63C1 group ($P = 0.0232$ and $P = 0.0452$, t-test) (Fig. 2A). This finding was also consistent with the results of virus titration (Fig. 2B).

We found another siRNA sequence, 3C2, which exhibited an inhibitory effect against FMDV, and we produced two recombinant adenoviruses that had either the U6 promoter or modified CMV promoter (Fig. 1B). We did not observe a statistically significant difference in the copy number of FMDV between the Ad-CMV3C2 and Ad-U63C2 treatment group at 48 h p.i. ($P > 0.05$, t-test, data not shown). Both Ad-U63C2 and Ad-CMV3C2 clearly showed an antiviral effect against FMDV, but the mixture of Ad-U62B-U63C1 and Ad-U63C2 (Ad-U62B-U63C1 + Ad-U63C2) or the mixture of Ad-U62B-U63C1 and Ad-CMV3C2 (Ad-U62B-U63C1 + Ad-CMV3C2) showed a decreased effect (Fig. 2C and D). When we analyzed the level of viral RNA at 48 h post-FMDV infection, Ad-U62B-U63C1 + Ad-U63C2 ($P = 0.0092$, t-test) and Ad-U62B-U63C1 + Ad-CMV3C2 ($P = 0.0495$, t-test) inoculation groups showed decreased antiviral effects when compared to the Ad-U62B-U63C1 group (Fig. 2C). The antiviral effects of Ad-U63C2 and Ad-CMV3C2 were lower than the effect of Ad-U62B-U63C1.

3.2. Enhanced antiviral effects by adenovirus simultaneously expressing three different shRNAs

We produced two recombinant adenoviruses that simultaneously express three shRNA sequences. One adenovirus expressed shRNAs with three U6 promoters and the other expressed shRNA with two U6 promoters and one CMV promoter (Fig. 1B). After inoculating adenovirus at 3×10^6 $TCID_{50}$, IBRS-2 cells were infected with FMDV. When we analyzed the level of FMDV RNA at 54 h post-FMDV infection, the adenovirus inoculation groups showed statistically significant inhibition of FMDV replication compared to the control group ($P < 0.05$, t-test) (Fig. 3A). An inoculating mixture of dual shRNA and single shRNA showed no improvement in the antiviral effect against FMDV compared to inoculation with the dual shRNA, Ad-U62B-U63C1. However, Ad-U62B-U63C1-CMV3C2

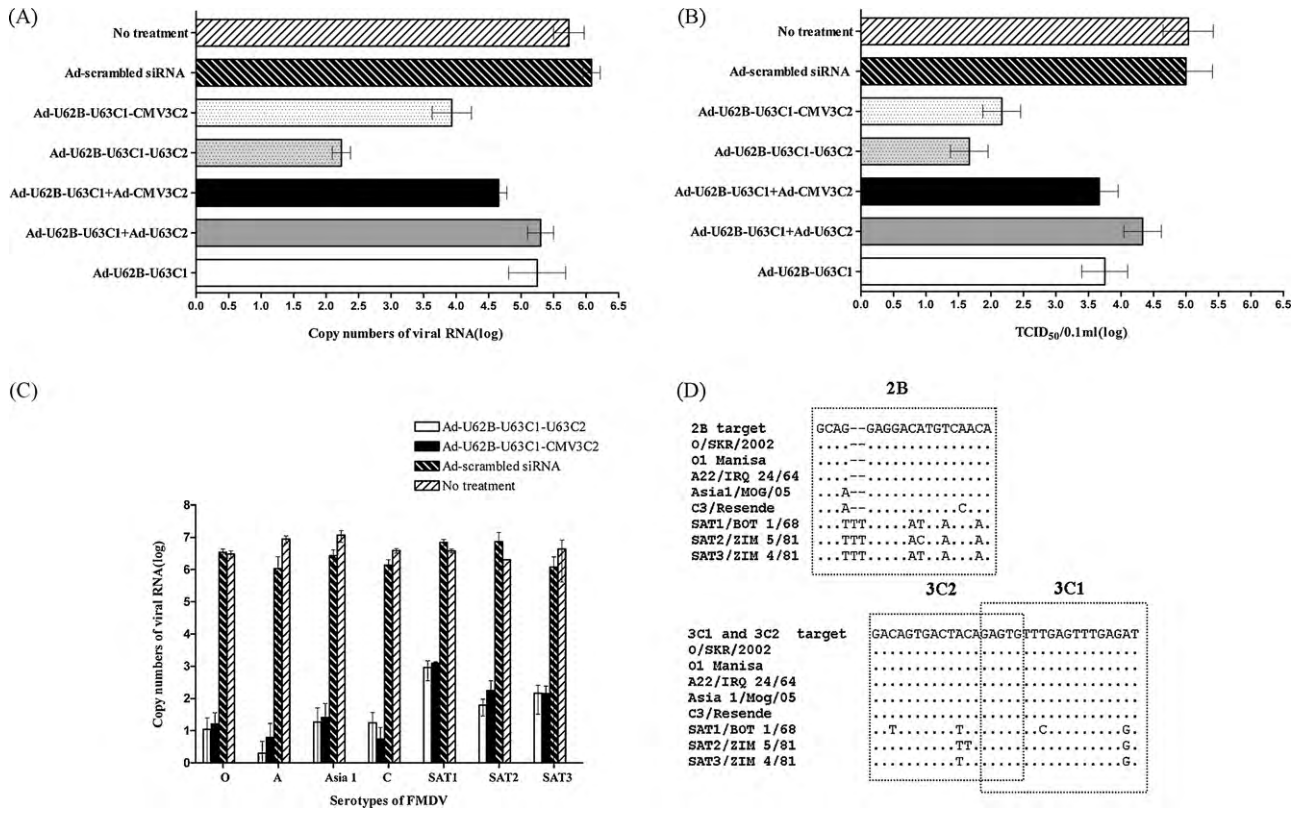

Fig. 3. Enhanced antiviral effects of adenoviruses simultaneously expressing three shRNAs in IBRS-2 cells. Cells were inoculated with Ad-shRNA at 3×10^6 $TCID_{50}$ (A and B) 1×10^7 $TCID_{50}$ (C). After 12 h, IBRS-2 cells were infected with 100 $TCID_{50}$ of FMDV O/SKR/2002 (A and B), O1 Manisa, Asia1/MOG/05, A22/IRQ 24/64, C3/Resende, SAT1/BOT 1/68, SAT2/ZIM 5/81and SAT3/ZIM 4/81 (C). Samples of supernatant were taken at 54 h following FMDV infection. The amount of FMDV RNA was determined by quantitative real-time RT-PCR (A and C). The titer of FMDV was determined by $TCID_{50}$ in IBRS-2 cells (B). (D) Nucleotide sequence alignment of siRNA target sites for seven serotypes of FMDV. Conserved sequences are shown as dots while gaps are shown as dashes. The target sequences of siRNAs are restricted by dot boxes.

($P = 0.0057$, *t*-test) and Ad-U62B-U63C1-U63C2 ($P = 0.0026$, *t*-test), which express three shRNAs, showed clear improvement in the antiviral effect against FMDV compared to Ad-U62B-U63C1. The Ad-U62B-U63C1-U63C2 exhibited a stronger antiviral effect than did Ad-U62B-U63C1-CMV3C2 ($P = 0.0028$, *t*-test).

The same results were obtained by the measurements of the virus titers (Fig. 3B). Ad-U62B-U63C1-CMV3C2 ($P = 0.0016$, *t*-test) and Ad-U62B-U63C1-U63C2 ($P = 0.0026$, *t*-test), the adenoviruses that expressed three shRNAs, showed clear improvement in their antiviral effect against FMDV compared to Ad-U62B-U63C1. In addition, Ad-U62B-U63C1-U63C2 showed a stronger antiviral effect than did Ad-U62B-U63C1-CMV3C2 ($P = 0.0919$, *t*-test).

3.3. Antiviral effects by adenovirus simultaneously expressing three different shRNAs against seven serotypes of FMDV and target sequence analysis

We observed antiviral effects when Ad-U62B-U63C1-CMV3C2 and Ad-U62B-U63C1-U63C2, the adenoviruses that express three shRNAs, were applied to O, A, Asia1, C, SAT1, SAT2, and SAT3 serotypes of FMDV (Fig. 3C). After inoculating the adenovirus at 1×10^7 $TCID_{50}$ on 96-well plates, we infected cells with FMDV. We analyzed the level of FMDV RNA at 54 h post-infection of FMDV, and then we observed the antiviral effect against the seven serotypes. When challenged, the replication of O, A, Asia1 and C types was significantly inhibited. Antiviral effects were also observed when SAT1, SAT2 and SAT3 were challenged (Fig. 3C). Stronger antiviral effects are seen in the results shown in Fig. 3C (1×10^7 $TCID_{50}$) than in the experiments shown in Fig. 3A and B (3×10^6 $TCID_{50}$), because we used the adenovirus at a higher titer.

We analyzed the siRNA target sequences of FMDV used in this study because we anticipated that antiviral effects of siRNAs would depend on sequence similarity (Fig. 3D). The target sequences 2B were completely conserved between the O and A serotypes of FMDV but had sequence variation in the following serotypes of FMDV: Asia1 (1 mismatch), C (2 mismatches), SAT1 (7 mismatches), SAT2 (7 mismatches) and SAT3 (7 mismatches). The target sequences of 3C1 and 3C2 had five overlapping nucleotide sequences. Their target sequences were conserved among the O, A, Asia1, and C serotypes of FMDV but had sequence variation in the SAT1 (4 mismatches), SAT2 (3 mismatches), and SAT3 (2 mismatches) serotypes of FMDV used in this study (Fig. 3D). Although there were mismatches in the sequences, the antiviral effects of the siRNAs were observed following challenges of the SAT1, SAT2, and SAT3 serotypes of FMDV. The number of mismatches in the viral target sequence was inversely proportionate to the silencing effect, and the application of multiple siRNAs had the advantage of inhibiting virus replication.

3.4. Assay for GFP silencing driven by modified CMV or U6 promoter

To demonstrate the silencing effects of the third siRNA driven U6 or modified CMV promoter, we replaced 3C2 siRNA with GFP in the plasmid expressing 2B, 3C1 and 3C2 siRNA (Entry-U62B-U63C1-U63C2 or Entry-U62B-U63C1-U63C2). The IBRS-2 cells were cotransfected with the GFP expressing plasmid with either GFP siRNA-expressing plasmids (Entry-U62B-U63C1-U6GFPsiRNA or Entry-U62B-U63C1-CMVGFPsiRNA) or 2B and 3C1 siRNA-expressing plasmid (Entry-U62B-U63C1). Cotransfection of the GFP plasmid with either GFP siRNA-expressing plasmids, resulted in

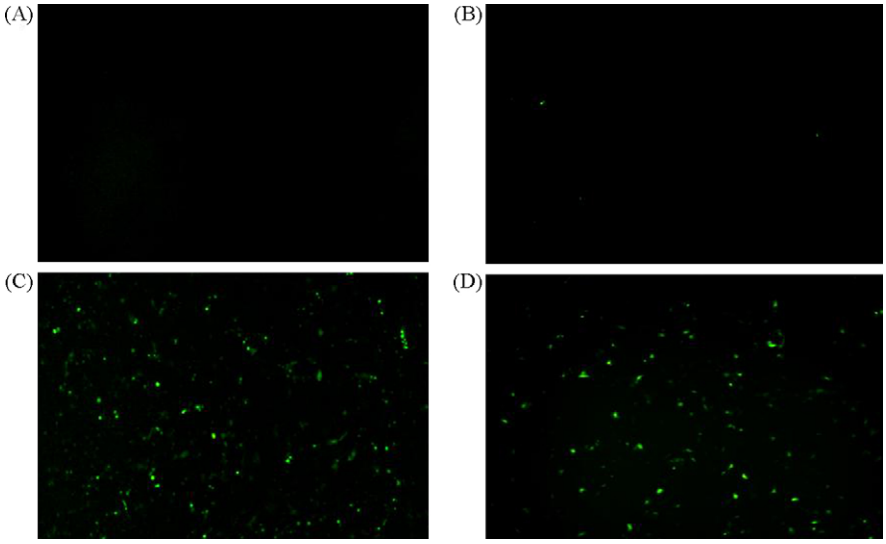

Fig. 4. Inhibition against GFP transient expression by GFP siRNA driven by U6 or modified CMV promoter. IBRS-2 cells were cotransfected with 4 μg Entry-U62B-U63C1-U6 GFPsiRNA (A), Entry-U62B-U63C1-CMV GFPsiRNA (B) or Entry-U62B-U63C1 as a control (C) and 0.5 μg of target plasmid, pIRES2-AcGFP (Clontech) were carried out with Lipofectamine 2000 (Invitrogen) as described by the Manufacturer. After 48 h, cells were examined microscopically for GFP expression. Images were collected with an Olympus l × 70 inverted microscope and an Olympus U-TVO, 5 × C-3 camera.

more than a 90% reduction in GFP expression relative to the control (Fig. 4). The control, Entry-U62B-U63C1, induced no significant reduction of GFP expression (Fig. 4C and D). The silencing efficiency of GFP siRNA driven by U6 was higher than that of GFP siRNA by modified CMV promoter in GFP signal. Cotransfection of GFP plasmid and Entry-U62B-U63C1-CMVGFPsiRNA resulted in a few cells with GFP signal, whereas Entry-U62B-U63C1-U6GFPsiRNA gave perfect reduction of the GFP signal at 48 h post-transfection (Fig. 4A and B).

3.5. Assays for cell toxicity

To determine whether siRNA expression could generate side effect in our systems, we assayed cell viability using the Guava ViaCount Reagent and Guava EasyCyte (Guava Technology). In IBRS-2 cells, the cell viability in the adenovirus control group was about 86% and the viability of the adenovirus expressing three shRNAs driven by three U6 promoters (46.3%) was significantly lower than for the control group (Table 3). However, no effect on cell viability was seen with the treatment with recombinant adenovirus expressing three shRNAs driven by two U6 and one CMV promoter.

3.6. Activation of IFN mRNA by adenovirus with three Pol III promoters

We measured the relative mRNA level of genes related to IFN pathway. An increase in IFN-α mRNA was not observed except in the positive control Ad-PoIFN-α treatment group (Fig. 5A). The IFN-β and PKR mRNAs were also induced in the treatment of the adenoviruses with the three U6 promoters and Ad-PoIFNα (Fig. 5B and C). The level of PKR mRNA was 9.6-fold (Ad-U62B-U63C1-U63C2) and 10.6-fold (Ad-PoIFNα), and the level of IFN-β mRNA was 58.5-fold (Ad-U62B-U63C1-U63C2) and 39.7-fold (Ad-PoIFNα) higher than that of the scrambled siRNA control. The level of OAS mRNA increased significantly in the treatment of adenoviruses with three U6 promoters and Ad-PoIFNα (Fig. 5D). However, in the case of the Ad-U62B-U63C1-CMV3C2 group, the levels of IFN-β, PKR and OAS mRNA were less than five fold higher than the Ad-scrambled siRNA group.

3.7. Antiviral effects in suckling mice by adenovirus expressing multiple shRNAs

To test the anti-FMDV effect of adenovirus-mediated shRNAs *in vivo*, we challenged suckling mice, pretreated with the adenoviruses by IP injection, with FMDV O/SKR/2002. Control mice challenged with 125 LD_{50} FMDV had an approximately 20% survival rate at 4 days post-challenge (dpc) and died within 6 or 7 dpc. Adenoviruses expressing short hairpins targeting non-structural protein regions 2B or 3C inhibited viral replication of FMDV in suckling mice ($P < 0.0001$, log-rank test) (Fig. 6A). The differences among survival rates of the Ad-CMV3C2, Ad-U63C2, Ad-U63C1, and Ad-U62B groups showed no statistical significance. The survival rate of the Ad-U62B-U63C1 group was higher than that of the Ad-U62B and Ad-U63C1 groups at 4, 5, and 6 dpc, respectively. However, survival rates were all at the same level by 7 dpc. The Ad-U62B-U63C1 group, which simultaneously expressed the 2B and 3C1 regions of FMDV showed no difference in survival rate when compared with the Ad-U63C1 group ($P = 0.8818$, log-rank test) and the Ad-U62B group ($P = 0.1369$, log-rank test). Treatment with a mixture of Ad-U62B-U63C1 and Ad-U63C2 (or Ad-CMV3C2) slightly enhanced the survival rate compared with

Table 3
Cell viability (%) after treatment with recombinant adenovirus expressing three shRNAs.

Treatment[a]	The numbers of total cells (×10^5/mL)	The numbers of viable cells (×10^5/mL)[b]	Viability (%)
Ad-U62B-U63C1-U63C2	4.23 ± 0.81	1.94 ± 0.21	46.3 ± 3.76
Ad-U62B-U63C1-CMV3C2	9.43 ± 0.75	8.30 ± 0.99	87.9 ± 3.47
Ad-scrambled siRNA	6.65 ± 1.41	5.77 ± 1.61	86.1 ± 5.89
No treatment	9.35 ± 0.83	8.05 ± 0.76	86.0 ± 0.55

[a] IBRS-2 cells were treated with recombinant adenovirus at 5×10^7 $TCID_{50}$ for 2 h and cells were suspended for assaying at 48 h post-treatment.
[b] The cells were stained using Guava ViaCount Reagent (Guava Technology) and viable cells were measured by Guava EasyCyte Plus system (Guava Technology).

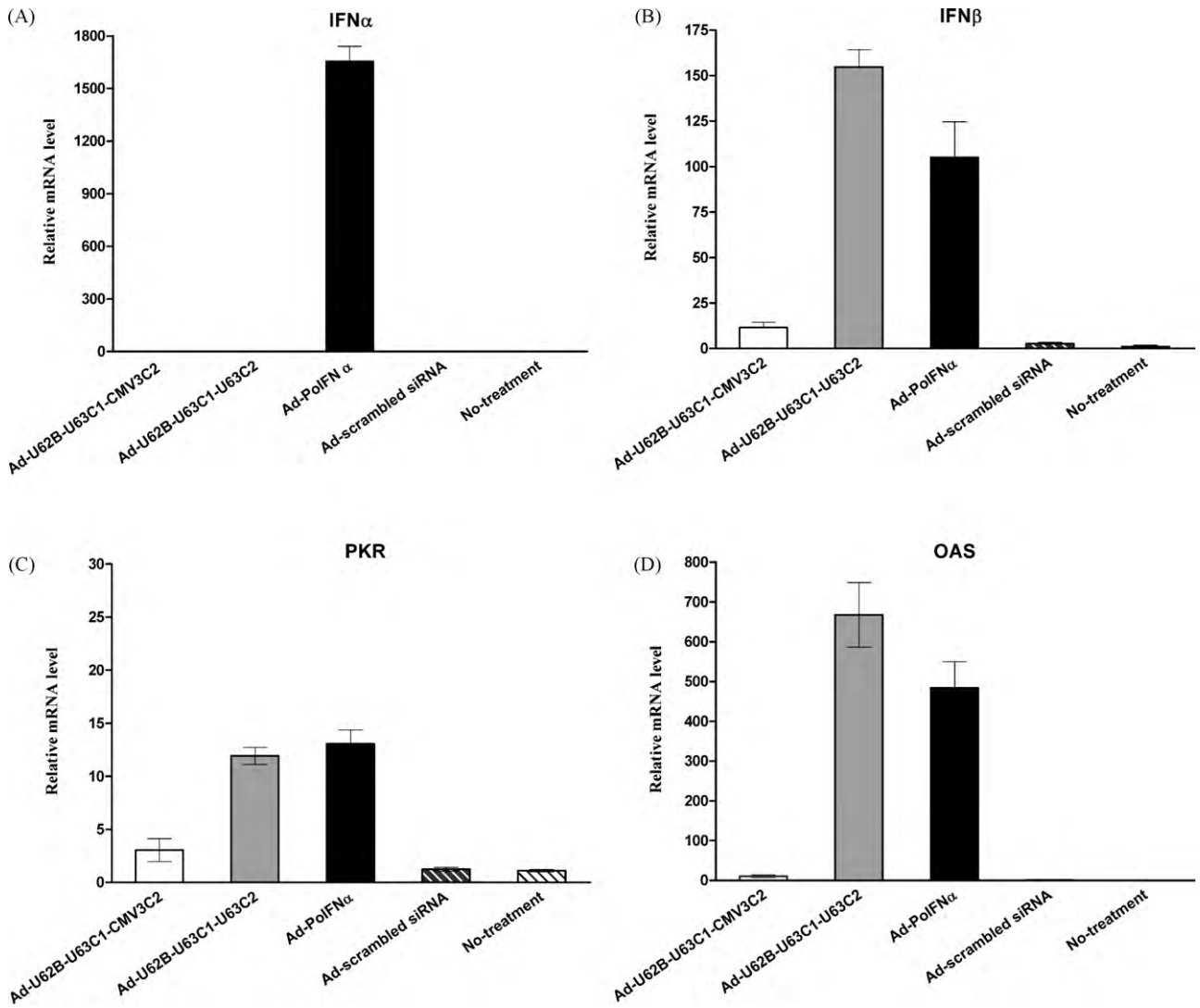

Fig. 5. Induced IFN responses by the adenovirus with Pol III promoters in IBRS-2 cells. Cells of 12-well plates were inoculated with recombinant adenoviruses at 8×10^7 $TCID_{50}$. Cells were collected at 12 h post-adenovirus infection and total RNA was extracted. Real-time RT-PCR was used to analyze the level of IFN-α (A), IFN-β (B), PKR (C) and OAS (D) mRNAs. GAPDH was used as an internal control and adenovirus expressing porcine IFNα was used as a positive control.

Ad-U62B-U63C1 at 7 dpc (Fig. 6B). However, the survival rate of mice given the mixture treatment was not statistically higher than that of those given Ad-U63C2, Ad-CMV3C2 and Ad-U62B-U63C1 ($P > 0.05$, log-rank test). When Ad-U62B-U63C1-U63C2 and Ad-U62B-U63C1-CMV3C2 (adenoviruses that simultaneously express three shRNAs) were used, the survival rate greatly improved compared to the group treated with Ad-U62B-U63C1 ($P < 0.0001$ and $P = 0.0001$, log-rank test). When the adenovirus expressing three shRNAs was used and O/SKR/2002 of 125 LD_{50} was challenged, the two groups maintained 90% or higher survival rates up to 7 dpc. To demonstrate the antiviral effects against other serotypes of FMDV with lower titer of adenovirus, we challenged suckling mice with FMDV A22/IRQ 24/64 or Asia1/MOG/05 after injection of 6×10^6 $TCID_{50}$ of adenoviruses. When Ad-U62B-U63C1-U63C2 was used in suckling mice challenged with A or Asia1 serotype of FMDV, the survival rate was about 90% up to 7 dpc. However, the survival rate of Ad-U62B-U63C1-CMV3C2 group was decreased by reducing adenoviral titer (Fig. 6C and D). The difference between the survival rate of Ad-U62B-U63C1-CMV3C2 group and Ad-U62B-U63C1-U63C2 challenged with A or Asia1 type of FMDV were statistically significant ($P = 0.0002$ and $P < 0.0001$, log-rank test).

4. Discussion

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, the silencing effects of siRNAs can be reduced by nucleotide mutation of viruses to avoid RNA interference (Gitlin et al., 2005). Researchers have described co-expression of multiple shRNAs from a single vector for human hepatitis C virus (HCV) and HIV (Grimm and Kay, 2007). The application of siRNA to FMDV in this study also required the simultaneous use of multiple siRNAs. We enhanced antiviral efficiency against FMDV by the *in vitro* and *in vivo* application of adenoviruses that simultaneously expressed three shRNAs and were driven by three U6 promoter or two U6 promoters and one CMV promoter. Moreover, we showed that the shRNA expression system driven by three separate U6 promoters was toxic in IBRS-2 cells. In our previous study, when we targeted two genes by mixing single shRNAs, we did not observe any improved inhibitory effects on virus replication either *in vitro* or *in vivo* (Kim et al., 2008). Therefore, we tried to improve the efficiency by producing adenoviruses that simultaneously expressed multiple shRNAs rather

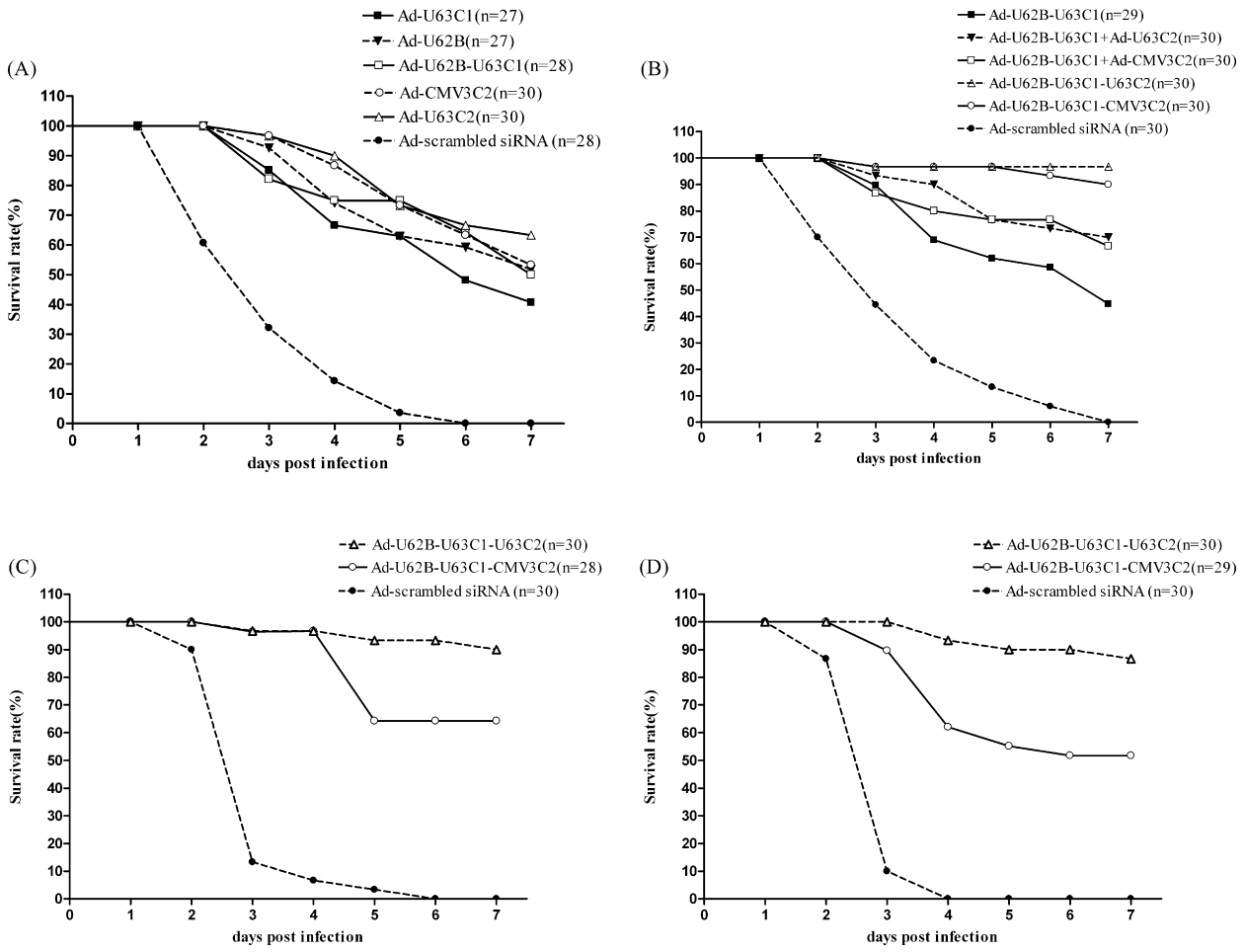

Fig. 6. Enhanced survival rates after FMDV challenge by treatment with recombinant adenovirus expressing three shRNAs in suckling mice. Suckling mice treated with adenoviruses expressing single shRNA or two different shRNAs at 6×10^7 $TCID_{50}$ (A), adenoviruses expressing three different shRNAs or adenovirus mixture at 6×10^7 $TCID_{50}$ (B) or adenoviruses expressing three different shRNAs at 6×10^6 $TCID_{50}$ (C and D) by three injections (6 h and 24 h before challenge and 2 days post-challenge). Suckling mice were challenged with 125 LD_{50} of O/SKR/2002 (A and B), A22/IRQ 24/64 (C), Asia1/MOG/05 (D) by intraperitoneal injection. Animals were monitored for 7 days.

than simply mixing single shRNAs. The simultaneous expression of two shRNAs in one plasmid has been shown to improve the antiviral effect against HBV (Wu et al., 2005). Furthermore, the treatment with a plasmid that simultaneously expressed shRNAs targeting HIV and HBV had antiviral effects against both viruses, with a similar efficiency to that of separate applications of single shRNAs (Wu et al., 2007). In this study, we also observed an improvement in the antiviral effect by an adenovirus expressing two different shRNAs (dual shRNA), compared with an adenovirus that expressed a single shRNA in IBRS-2 cells (Fig. 2A and B).

The present study was designed to enhance the antiviral effects of an adenovirus expressing two different shRNAs by using three shRNAs. In the first attempt, we mixed an adenovirus expressing dual shRNAs with one expressing a single shRNA. We observed no improvement of antiviral effects in the IBRS-2 cell; in fact, the antiviral effect decreased (Fig. 2C and D). In the second attempt, we produced adenoviruses expressing three shRNAs using three promoters, and we observed a highly improved anti-FMDV effect. This result showed that the strategy of expressing siRNAs simultaneously in one adenovirus is more effective than the strategy of mixing siRNAs (Fig. 3A and B). When only siRNAs are mixed, their effects decreased because there were high efficient siRNA and low efficient one (Fig. 2C and D). A similar decrease in efficacy was seen when four siRNAs were pooled and treated against Venezuelan equine encephalitis virus (O'Brien, 2007). Other researchers compared the application of one plasmid in which three shRNAs were expressed with the application of several pooled shRNAs. They observed that the antiviral effect of the one plasmid expressing three shRNAs against HBV was much stronger (Chen and Mahato, 2008). Recent reports have also shown that multiple targeting siRNAs had synergistic effects, demonstrating that a RNAi vector that included four target sites had significantly stronger knockdown effects against the murine CypA gene (Song et al., 2008). Furthermore, vector-based multiple shRNAs have been shown to have great potential for inhibiting target genes and act in a dose-dependent manner as shown by placing copies of shRNA (Gonzalez et al., 2005). An adenovirus with three promoters could express about three times the number of shRNA copies; thus, multiple targeting RNAis might have synergistic effects.

We observed antiviral effects against A, Asia1, C, SAT1, SAT2 and SAT3 serotypes, in addition to the O type of FMDV, when an adenovirus simultaneously expressed three shRNAs targeting the non-structural protein regions (Fig. 3C). We selected siRNA sequences targeting 2B and 3C region because these regions are the most conserved in several types of FMDV (Carrillo et al., 2005). The sequence alignments of siRNA targets showed that the number of mismatches in the viral target sequence was inversely proportional to the silencing effect (Fig. 3D). Similar studies have suggested that silencing activity varies depending on whether mismatches are present in the target sequence or in the position of mismatch (Pusch et al., 2003; Sabariegos et al., 2006). 3C1 and 3C2 target overlapping sequences (Fig. 3D). However, we did not observe negative

or synergistic effects due to overlapping in this study (Fig. 3A and B).

In order to express three different shRNAs, we attempted to use the modified CMV promoter which is an RNA Pol II promoter, together with two U6 promoters which are an RNA Pol III promoter. Pol III promoters (including U6) have been widely used to mediate the high-level expression of shRNAs. However, shRNA expression from RNA Pol III promoters has been shown to induce cell toxicity and lethality in adult mice (Grimm et al., 2006). We were therefore concerned about the *in vivo* and *in vitro* toxicity of expressing three U6 promoters. We anticipated that different promoters might also cause a difference in efficacy between *in vitro* and *in vivo* expression. The difference in antiviral effect against HBV of the plasmid that had RNA Pol III and Pol II promoters has been reported previously in the HepG 2.2.15 cells and in Balb/c mice (Ren et al., 2007). However, we could not observe significant differences in efficacy of the 3C2 shRNA driven by U6 and modified CMV promoter *in vitro* and *in vivo* (Fig. 2C and D and 6A).

In our study, the adenovirus with three separate U6 promoters showed slightly improved efficacy and did have some cytotoxic effects in IBRS-2 cells, compared with the adenovirus with combination of two U6 promoters and one CMV promoter (Fig. 3A and B; Table 3). The adenovirus expressing two shRNAs driven by two U6 promoters did not show cytotoxicity in our study (data not shown) and a plasmid encoding three shRNAs driven by a single U6 promoter has been reported to not induce apoptotic changes (Chen and Mahato, 2008). In a previous study, there was no data as to whether shRNAs driven by three H1 promoters or U6, 75K and H1 promoters induce cell toxicity; however, the researchers used only single U6 promoter (ter Brake et al., 2008). We observed that three shRNAs, expression driven by three separate U6 promoters, were toxic to IBRS-2 cells. The reason for increased toxicity might be that the shRNA expressed Pol III promoter-induced interference of the cellular RNAi pathway (Grimm et al., 2006). In this study, we demonstrated that the adenovirus expressing multiple shRNAs driven by three U6 promoters induce toxicity. This can be explained by the fact that the siRNAs are made by three U6 promoters and type I interferon activates cells and activate interferon pathways in cells (Fig. 5). The induction of IFN-β, PKR, and OAS mRNAs were observed following treatment with adenoviruses with three U6 promoters and with the positive control, although the previous study has suggested that IBRS-2 cells had intact IFN-α/β induction after FMDV infection (Chinsangaram et al., 2001). We also observed that the cell proliferation decreased after treatment with adenoviruses with three U6 promoters (data not shown). These observations might represent indirect evidence of IFN induction (Abbas and Lichtman, 2003). However, an increase in IFN-α mRNA was not observed except in the positive control Ad-PoIFN-α treatment group (Fig. 5). Epithelial cells, including IBRS-2 cells, might show a high level of IFN-β and a slight increase in IFN-α because IFN-β is produced by many cells, including fibroblasts and epithelial cells, while, IFN-α is primarily produced by mononuclear phagocytes (Abbas and Lichtman, 2003). Elevated apoptosis has been observed in primary human lymphocytes following stable shRNA expression via a lentivirus with a single U6 promoter but not a H1 promoter and they said that lower efficiency (expression level) of H1 promoter had advantages in safety. However, there was no data regarding IFN response (An et al., 2006). We suggest that a high level of shRNA expression by U6 promoters could induce IFN response and that cell toxicity is related not only to the saturation of miRNA exporting factors (Grimm et al., 2006) but also to the IFN response. The off-target effects were concentration-dependent as well as siRNA sequence dependent (Persengiev et al., 2004).

The two adenoviruses expressing three shRNAs showed no statistical significance in terms of differences in the survival rates of suckling mice treated with recombinant adenovirus at 6×10^7 $TCID_{50}$ (Fig. 6). However, in the case of suckling mice treated with recombinant adenoviruses at 6×10^6 $TCID_{50}$, the survival rate of the Ad-U62B-U63C1-U63C2 group was significantly higher. The survival rate was approximately 90%, although lower titer and other serotypes (Asia1 or A) of challenging virus (Fig. 6C and D). We suggested that Ad-U62B-U63C1-U63C2 group had a stronger antiviral effect because expression efficiency of the U6 promoter was very high and 3C2 siRNA played an important role in the silencing of the adenoviruses expressing 3 siRNAs. Therefore, the antiviral effect of Ad-U62B-U63C1-CMV3C2 might be decreased rapidly by reducing the adenoviral titer. The survival rate of mice exposed to multiple shRNAs driven by three U6 promoters indicated that there were no toxic effects. In addition to, it indicated strong antiviral effects *in vivo*. Possible reasons for a lack of toxicity may be that the suckling mice model lacks IFN responses (Ryman et al., 2000), that recombinant virus was not treated at high multiplicity of infection (MOI) as the experiments in cells, or that the efficiency of siRNA expression was decreased *in vivo*. Therefore, we suggested that the suckling mice treated with recombinant adenoviruses were not affected by overexpression of shRNA.

In conclusion, this study demonstrated that adenoviruses simultaneously expressing three different shRNAs had significantly enhanced antiviral effects and were able to inhibit replication in seven serotypes of FMDV. In addition, the approach that uses multiple shRNAs driven by combination of Pol II and Pol III promoters is probably a more desirable strategy for antiviral efficiency without cell toxicity. However, if we use the adenovirus three U6 promoters in natural host models such as pigs and cattle, the toxicity might be negligible.

Acknowledgements

We thank Mr. Min-Woo Bang for assistance with gene cloning and animal experiments. This work was supported by the National Veterinary Research and Quarantine Service (NVRQS), Republic of Korea.

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