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Specific small interfering RNAs-mediated inhibition of replication of porcine encephalomyocarditis virus in BHK-21 cells

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Abstract

Encephalomyocarditis virus (EMCV) is recognized as a pathogen inducing acute myocarditis and sudden death in preweaned piglets and severe reproductive failure in sows. In this study, eight specific small interfering RNA (siRNA) duplexes targeting different genomic regions of EMCV BJC3 were designed and their ability to inhibit virus replication in BHK-21 cells was investigated. The results showed that BHK-21 cells transfected with siRNA duplexes to 2C gene (JH-4666, BJC-1739), 2B gene (BJC-807), 3C gene (BJC-2363) and 3D gene (BJC-3269) were specifically resistant to EMCV infection when exposed to 500 times the 50% cell culture infective dose ($CCID_{50}$) of EMCV. The levels of the 3D gene in the transfected cells were obviously decreased. IFA and Western blotting analysis confirmed that the expression of VP1 protein in cell culture transfected with the siRNAs was apparently reduced. Of the five siRNAs, JH-4666, BJC-2363 and BJC-3269 were the most effective. Combination of the siRNA duplexes enhanced the inhibition of EMCV replication. Our data indicated that specific siRNAs are able to inhibit the replication of porcine encephalomyocarditis virus in BHK-21 cells, suggesting that RNAi might provide a new approach to prevent EMCV infection.

Keywords: Encephalomyocarditis virus (EMCV); RNA interference (RNAi); Small interfering RNA (siRNA); EMCV BJC3 isolate; BHK-21 cells; Inhibition; Replication

1. Introduction

Encephalomyocarditis virus (EMCV) is a member of the genus Cardiovirus, family Picornaviridae. The genome is a single-stranded, positive-sense RNA of approximately 7.8 kb with a unique large open reading frame (ORF) (Palmenberg et al., 1984). Similar to other picornaviruses, the genome of EMCV is covalently linked at the 5′ end to a protein called virion protein, genome-linked (VPg) (Flanegan et al., 1977), in the 5′untranslated region (UTR) it contains the internal ribosomal entry site (IRES). The 3′UTR ends with a short heterogeneous poly(A) tail. Among the genes of EMCV, the 3D gene that encodes the RNA-dependent RNA polymerase is the most conserved. The genomic RNA serves as a template for viral RNA transcription to synthesize more copies of positive genomic RNA through a negative intermediate, and also acts as an mRNA template for translation of a single polyprotein that is post-translationally processed primarily by proteases 2A and 3C to produce individual structural proteins including VP1, VP2, VP3 and VP4 protein and nonstructural proteins.

Since fatal disease of swine caused by EMCV was first described in 1958 (Murnane et al., 1960), the virus has been recognized worldwide as a pathogen that can infect many host species including pigs, rodents, cattle, elephants, raccoons, marsupials, and primates such as baboons, monkeys, chimpanzees (Gelmetti et al., 2006; Koenen, 2006; LaRue et al., 2003). Some evidences of EMCV infection and seroprevalences in humans imply that the virus imposes potential significance in public health (Deutz et al., 2003a,b; Juncker-Voss et al., 2004; Kirkland et al., 1989). The pig is considered the most commonly

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and severely infected domestic animal species (Acland and Littlejohns, 1975; Billinis et al., 1999). EMCV infection could cause acute myocarditis and sudden death in preweaned piglets (Billinis et al., 1999; Gelmetti et al., 2006) and severe reproductive failure in sows (Dea et al., 1991; Koenen et al., 1994; Love and Grewal, 1986), resulting in severer economic losses for swine production (Acland and Littlejohns, 1975; Koenen et al., 1999). Recently, EMCV infection in pigs was shown to be prevalent in many intensive swine farms (J.L. Zhang et al., 2007), and EMCV isolates have been identified and characterized in China (Ge et al., 2007; G.Q. Zhang et al., 2007). Moreover, EMCV may be a potential pathogen for recipients in xenotransplantation (Brewer et al., 2003; Denis et al., 2006). Xenotransplantation research has focused on using pig tissues and cells, including heart valves, skin, hepatocytes and neural cells to treat various diseases in humans (Lanza and Cooper, 1998). Porcine islet cells (PICs), which are susceptible to porcine EMCV, are used in clinical trials for treatment of type I diabetes in humans. Although EMCV infection does not appear to affect insulin production by PICs, infected xenografts can transmit the virus to recipient animals, resulting in severe disease (Brewer et al., 2004). Recently, transplantation of myocardial and pancreatic tissues from acutely infected pigs transmitted the virus to recipient mice, resulting in acute fatal EMCV disease, suggesting that the virus may provide a risk in pig to human transplantation (Brewer et al., 2003). Therefore, preventing EMCV infection in pigs is very important for swine production and future xenotransplantation in human.

Vaccination is considered as one of the effective strategies for controlling EMCV infection. Although traditional and novel vaccines associated with EMCV have been reported (Hunter et al., 1998; Osorio et al., 1996; Sin et al., 1997; Suh et al., 2001), these vaccines have not been applied widely in practice. Moreover, it has been shown that the vaccine was effective in pigs as early as 7 days post-exposure (Osorio et al., 1996), but did not provide early protection against EMCV infection in piglets. Thus, developing a new rapid-acting and effective antiviral strategy against EMCV infection in piglets will be worthy to be considered. Recent studies have shown that RNA interference (RNAi) is active and effective against infection of animal pathogenic viruses (Chen et al., 2006; He et al., 2007; Huang et al., 2006; Kahana et al., 2004; Liu et al., 2005; H. Zhou et al., 2007; J.F. Zhou et al., 2007). These data support the use of RNAi as a novel antiviral therapy in animals. In this study, we investigated the ability of short interfering RNAs (siRNAs)-mediated RNA interference for EMCV replication in baby hamster kidney (BHK) cells by using siRNAs targeting different regions of the genomic RNA of EMCV.

2. Materials and methods

2.1. Cell cultures and viruses stock

Baby hamster kidney cells (BHK-21) and MARC-145 cells (China Institute of Veterinary Drug control) were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO^{TM}, Invitrogen Corporation) supplemented with 10% (v/v) bovine calf serum (GIBCO^{TM}, Invitrogen Corporation), 10 mM Hepes, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (pH 7.4). BHK-21 cells were used for propagation and challenge of EMCV. EMCV BJC3 was isolated and identified in our laboratory (Ge et al., 2007; G.Q. Zhang et al., 2007). Porcine reproductive and respiratory syndrome virus (PRRSV) BJ-4 strain (North American type) was characterized in our laboratory (Yang et al., 1997) was used as control virus in this study. Determination of the virus titers was conducted by a standard 50% cell culture infective doses ($CCID_{50}$) assay using the Reed and Muench method (Reed and Muench, 1938).

2.2. Selection and preparation of siRNAs

According to the genomic sequences of EMCV BJC3 (GenBank accession no. DQ464062), eight siRNAs corresponding to viral capsid protein 1A and 1C, viral protease 2A and 3C, viral nonstructural protein 2B and 2C, and RNA-dependent RNA polymerase 3D were designed by Ambion's siRNA "Target Finder and Design Tool" available at http://www.ambion.com/techlib/misc/siRNA_finder. Specificity of these sequences was verified by BLAST search of the National Center for Biotechnology Information's expressed sequence tag library. All siRNA duplexes consisted of two complementary 21-nucleotide RNA strand with 3′dTdT overhangs and were chemically synthesized (Shanghai GenePharma Co. Ltd.). Scrambled siRNA duplex (Shanghai GenePharma Co. Ltd.) with no sequence similarity to the genome of EMCV was used as negative control to ensure the specificity of the observed effects. The sequences of the siRNAs used in the current studies are summarized in Table 1.

2.3. Transfection of siRNAs and virus infection

BHK-21 cells were trypsinized and seeded in 12-well plates (Costar, Corning Incorporated, U.S.A.) at 4×10^5 cells per well in DMEM containing 2% bovine calf serum 1 day before transfection and were maintained in a 5% CO_2 humidified incubator at 37 °C. Then, the cells were transfected under optimal conditions. Briefly, when the cells reached about 70–80% confluence, they were washed and overlaid with transfection complexes containing 2 μg chemically synthesized siRNA duplexes in 100 μl of OPTI-MEM medium (GIBCO^{TM}) mixed with DMRIE-C Reagent (Invitrogen Corporation) according to the Manufacturer's instructions. A mock control (the cells transfected by transfection reagent with no siRNA) was used to check for any cytotoxicity arising from the reagent, and FITC negative control siRNA duplex was used to check the transfection efficiency by BD FACSCalibur^{TM} (Becton, Dickinson and Company). All 12-well plates were filled with 1 ml medium per well. After 4 h post-transfection, the cells were infected with 500 $CCID_{50}$ of EMCV BJC3 per well. After incubation at 37 °C, 5% CO_2 humidified incubator for 30 min, the cells were washed twice and cultured with DMEM containing 2% bovine calf serum. Cells were examined with an Olympus microscope (Olympus, U-LH 50HG, Japan), and images were collected with an Olym-

Table 1
Targeted genes, positions and sequences of siRNAs used in this study

siRNA	Targeted gene of EMCV	Position[a]	Sequence
JH-210	1A	1037–1055	5′-CCUCCUCAGACAAGAAUAAdTdT-3′
JH-1237	1C	2064–2102	5′-CAGCACAGUGCCUAUUUAUdTdT-3′
JH-2932	2A	3759–3777	5′-GGCGGUUCUAAGAGCAGAAdTdT-3′
JH-4666	2C	5493–5511	5′-GGAGCGUAACUCUGUGUUUdTdT-3′
BJC-807	2B	4336–4354	5′-GCAGCUAAGUUCAAGACAAdTdT-3′
BJC-1739	2C	5268–5286	5′-CAGAGAUAACCGAACUAAAdTdT-3′
BJC-2363	3C	5892–5910	5′-CGCCCGCUCUACUGUUAAAdTdT-3′
BJC-3269	3D	6798–6816	5′-GGAUGAGCUUAGACCGAUAdTdT-3′
Scramble siRNA[b]			5′-CCUCCUCAGACAAGAAUAAdTdT-3′

[a] Nucleotide positions of siRNA according to the genomic RNA of EMCV BJC3 (GenBank accession no. DQ464062).
[b] Scrambled siRNA with no significant sequence similarity to the targeted genes of EMCV.

pus video camera. Supernatants of cell culture and cell lysates were collected at different time post-infection.

In order to analyze whether a pool of siRNA duplexes targeting different genomic regions of EMCV BJC3 could enhance the inhibitory effect, 0.5 μg of each of the siRNA duplexes was mixed together. BHK-21 cells were transfected and infected as described above. To further analyze whether the inhibition of siRNA duplexes was dose-dependent, we selected the efficient siRNAs. BHK-21 cells seeded in 12-well plates were transfected with 0.4, 2 and 10 μg siRNA duplexes, respectively, followed by EMCV infection as described above and supernatants of the cell cultures were collected at 18 h post-infection.

To detect whether the inhibition of siRNA duplexes was mediated by an off-target effect, PRRSV, a single-stranded and positive-sense RNA virus, was used as virus control. MARC-145 cells seeded in 12-well plates were transfected with 2 μg siRNA duplexes and infected with 500 $CCID_{50}$ of PRRSV BJ-4 per well as described above. Supernatants collected at 48 h post-infection were prepared for viral titration after twice freeze-thawing.

2.4. Virus titration

BHK-21 cells were seeded in 96-well plates (Costar, Corning Incorporated) with 5×10^4 cells per well in DMEM containing 10% bovine calf serum 1 day before infection. Supernatants obtained at 18, 24, 30 and 36 h post-infection were 10-fold serially diluted and added to wells, 100 μl per well in triplicate, then the virus titers were determined as $CCID_{50}$ by the Reed and Muench method (Reed and Muench, 1938).

2.5. Real-time quantitative PCR analysis

To more accurately detect EMCV BJC3 replication in the transfected cells at 18 h post-infection, total RNAs were extracted from 250 μl of supernatant sample of the cell lysates using Trizol® reagent (Invitrogen Corporation), digested with Rnase-free DNase I (RNase Free) (TaKaRa Bio Technology Co. Ltd., Dalian, China) and then reverse transcribed to cDNA. One microliter of the cDNA product was used as the template for real-time quantitative PCR which was performed in a DNA Engine Opticon™ 2 Real-Time PCR Detector (MJ Research Inc., Waltham, MA) in a 20 μl reaction volume using SYBR® Green Realtime PCR Master Mix (TOYOBA Co. Ltd., Osaka, Japan), according to the Manufacturer's recommendations. The following two pairs of primers were used: 3DP1, 5′ GGTGAGAGCAAGCCT CGCAAAGACAG 3′ and 3DP2, 5′ CCCTACCTCACGGAATGGGGCAAA G 3′ for 3D gene of EMCV; β-Actin-F, 5′ TCATCACTATTGGCAACGAGC 3′and β-Actin-R, 5′ AACAGTCCGCCTAGAAGCAC 3′ for β-actin gene of BHK-21 cells. The reaction mixture contained 10 μl SYBR® Green Realtime PCR Master Mix (2×), 0.4 μl of 10 μM sense primer and 0.4 μl of 10 μM anti-sense primer. The Real-time quantitative PCR was performed at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Fluorescence was measured following each cycle and analyzed by MJ Opticon™ analysis software Ver 3.1 (MJ Research Inc., Waltham, MA). The relative amounts of 3D gene mRNAs of EMCV were normalized for loading differences by β-actin gene mRNA. The copies of 3D gene mRNAs were quantified by comparison with a standard curve derived from known amounts of plasmids containing EMCV 3D gene (pMD-18T-3D). All samples were run in triplicate.

2.6. Indirect immunofluorescence assay

The expression of viral capsid protein of EMCV was ascertained by an indirect immunofluorescence assay using a monoclonal antibody (C11) against EMCV VP1 protein. The transfected cells were fixed with pre-chilled alcohol at 12 h post-infection, and 100 μl of C11 ascites fluid at a dilution of 1:50 was added to each well and incubated for 1 h at 37 °C in a humidified compartment. Then, 50 μl of fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG(H + L) (Sigma–Aldrich, St. Louis, MO) was added per well at dilution of 1:100 and incubated for 30 min at 37 °C, and finally visualized with an Olympus fluorescence microscope (Olympus, U-LH 50HG, Japan).

2.7. Western blotting analysis

To compare the differences in viral capsid VP1 expression of EMCV after treatment with each interfering RNA, the transfected cell were collected and washed twice with phosphate-buffered saline (PBS pH 7.4) at 18 h post-infection, subsequently lysed with lysis buffer. Ten microliters of super-

natant samples of the transfected cell lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred to nitrocellulose membrane (Millipore). The membrane was blocked with 10% skim milk for 1 h at 37 °C, then probed with C11 ascites fluid at a dilution of 1:20. To normalize protein loading, the membrane was simultaneously incubated with rabbit polyclonal antibody against β-actin (Santa Cruz Biotechnology, Inc.) at a dilution of 1:200, followed by incubation with HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG at a dilution of 1:10,000 (Sigma–Aldrich, St. Louis, MO). Immunoblotting was examined by SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the Manufacturer's instruction, and the membrane was exposed to X-ray films (Kodak) which were developed with the Alpha Imager (Alpha Imager™).

2.8. Statistical analysis

Results were presented as mean ± standard deviation. The significance of variability among the experimental groups was determined by one-way or two-way ANOVA using of Graphad Prism® Version 4.0 software. Differences among experimental groups were considered statistically significant if $P < 0.05$.

3. Results

3.1. Inhibition of EMCV replication in BHK-21 cells by individual synthetic siRNAs

The efficacy of synthetic siRNAs to inhibit EMCV replication in BHK-21 cells was first analyzed. BHK-21 cells were transfected with 2 μg of individual siRNA duplexes (JH-210, JH-1237, JH-2932, JH-4666, BJC-807, BJC-1739, BJC-2363 and BJC-3269). Each assay was run in triplicate in a 12-well plate. Simultaneously, scrambled siRNA duplexes were used as a negative control for off-target effects and mock cells treated with transfection reagent without siRNA was used as control for checking any cytotoxicity arising from the reagent. The transfection efficiency of FITC negative control siRNA duplex was determined to be about 40% by flow cytometry. The cytopathic effect (CPE) was observed microscopically starting at 6 h post-infection with EMCV BJC3. For scrambled siRNA duplex and mock transfected cells, obvious morphological changes including cellular nucleus shrinkage and cell lysis initiated appearance at 12 h post-infection and all cells were lysed at 18 h post-infection. In contrast, the normal cell control monolayer remained intact at all time points examined. BHK-21 cells transfected with the synthetic siRNAs JH-4666, BJC-807, BJC-1739, BJC-2363, BJC-3269 obviously reduced susceptibility to EMCV infection when exposed to 500 $TCID_{50}$ of EMCV; among them, JH-4666, BJC-2363 and BJC-3269 were the most effective, showing that the appearance of CPE induced by EMCV was remarkably delayed until 24 h post-infection (data not shown), compared to the scrambled siRNA duplex and mock transfected cells. This implies that the target genes of these siRNAs may be necessary for EMCV life cycle. JH-210, JH-1237 and JH-2932 had weak or no effects for inhibition of CPE induced by EMCV. CPE of BHK-21 cells at 18 h post-infection is shown in Fig. 1.

The virus titers of supernatant samples collected at 18, 24, 30 and 36 h post-infection were determined. Our data indicated that the virus titers in the infected cells transfected with the synthetic siRNAs JH-4666, BJC-807, BJC-1739, BJC-2363, BJC-3269 were reduced by 166-, 46.5-, 40-, 215-, 87-fold at 18 h post-infection, respectively, compared to the cells transfected with scrambled siRNA duplex ($P < 0.001$) (Fig. 2A). No significant inhibitions were observed in the cells transfected with scrambled siRNA duplex and mock cells ($P > 0.05$). No significant differences were exhibited between the virus titers in the infected cells transfected with the synthetic siRNAs and those in the cells transfected with scrambled siRNA duplex and mock cells at 30 and 36 h post-infection (data not shown). At the same time, RNA expression level for 3D gene of EMCV BJC3 in the transfected cells at 18 h post-infection was measured by real-time quantitative PCR. The results indicated that the mRNA level of 3D gene in the cells transfected with JH-4666, BJC-807, BJC-1739, BJC-2363 and BJC-3269 decreased by 214-, 32-, 22-, 218-, 85-fold, respectively, compared to the cells transfected with scrambled siRNA duplex ($P < 0.001$) (Fig. 2B), showing that the transcription level of the 3D gene was paralleled with the virus titers in the transfected cells.

VP1 protein expressions in the transfected cells were analyzed by IFA and Western blotting using a monoclonal antibody (C11) against EMCV. IFA analysis showed that the infected cells transfected with JH-4666, BJC-807, BJC-1739, BJC-2363 and BJC-3269 exhibited less positive signal compared to the cells transfected with scrambled siRNAs (Fig. 3A). Western blotting analysis revealed that the cells transfected with BJC-807, BJC-1739 displayed a weak band of 30 kDa protein, and the cells transfected with JH-4666, BJC-2363 and BJC-3269 had an almost invisible band, while the cells transfected with JH-2932 and scrambled siRNAs showed obvious bands (Fig. 3B).

3.2. Enhanced inhibition effects of EMCV BJC3 replication in BHK-21 cells by a pool of siRNA duplexes

Enhanced inhibitory effects of a pool of siRNA duplexes were analyzed. BHK-21 cells were transfected with a mixture of 0.5 μg of each of the siRNA duplexes and infected with 500 $CCID_{50}$ of EMCV BJC3. CPE analysis indicated that the cells transfected with a pool of siRNA duplexes significantly reduced susceptibility to EMCV infection compared to single siRNA duplex transfected cells, and remarkably delayed the appearance of the CPE induced by EMCV (Fig. 1). The virus titer of supernatants of the transfected cells collected at 18 h post-infection decreased by 359-fold ($P < 0.001$) (Fig. 2A), and the RNA expression level of EMCV 3D gene lowered by 335-fold ($P < 0.001$) (Fig. 2B) relative to the scrambled transfected cells, which was obviously lower than in the single siRNA duplex-transfected cells. VP1 protein expression was also confirmed by IFA (Fig. 3A) and Western blotting (Fig. 3B). The results indicated that the pool of siRNA duplexes could enhance the inhibition of EMCV replication.

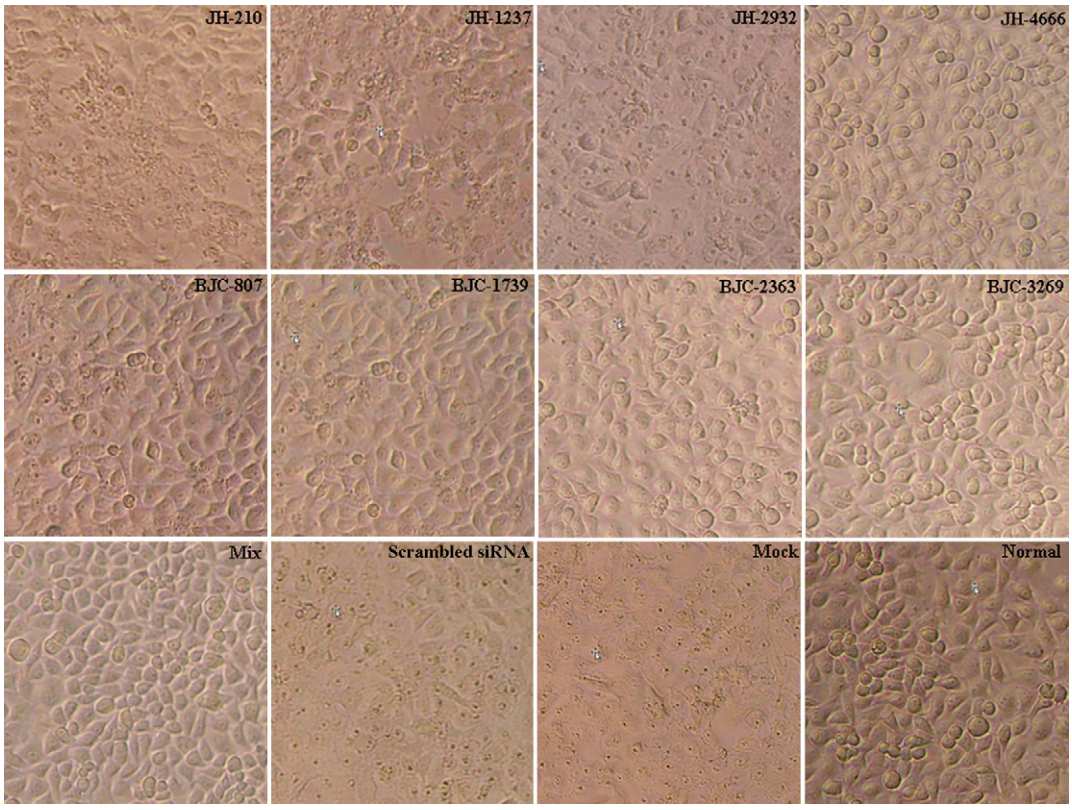

Fig. 1. EMCV-induced CPE in BHK-21 cells transfected with the siRNAs. BHK-21 cells were transfected with 2 μg of individual siRNAs (JH-210, JH-1237, JH-2932, JH-4666, BJC-807, BJC-1739, BJC-2363, BJC-3269), mixture of siRNAs (Mix), scrambled siRNAs and mock control, respectively. The cells were infected with 500 $CCID_{50}$ of EMCV BJC3 at 4 h post-transfection. CPE was examined at 18 h post-infection.

3.3. Dose-dependent inhibition of EMCV replication in BHK-21 cells by the siRNAs

The inhibition efficiency induced by different dose of siRNA was investigated. BHK-21 cells were transfected with 0.4, 2 and 10 μg of JH-4666, BJC-2363, BJC-3269, respectively, and followed by infection of EMCV BJC3. The virus titers of supernatants collected at 18 h post-infection were assayed. Data showed that the virus titers decreased as the dose of siRNA duplexes increased (Fig. 4). The difference of the virus titers between 0.4 and 2 μg of siRNAs was significant ($P < 0.05$), indicating the inhibition efficiency induced by the siRNAs for EMCV replication in BHK-21 cells was dose-dependent.

3.4. No off-target inhibition of PRRSV replication in MARC-145 cells by the siRNAs

No significant differences were observed among the virus titers of supernatants obtained from each well of PRRSV infected cells (Fig. 5), indicating that no off-target inhibition of PRRSV replication in MARC-145 cells by the siRNAs, and their inhibition efficiency was specific for EMCV.

4. Discussion

RNAi is a biological process of sequence-specific, post-transcriptional gene silencing that is initiated by double stranded RNA molecule with 19–27 nt duplexes in the cytoplasm of eukaryotic cells (Agami, 2002). Since it was found that experimental introduction of exogenous double-stranded RNA into *C. elegans* and mammalian cells was able to specifically silence homologous mRNA (Fire et al., 1998; Elbashir et al., 2001), RNAi technology has been demonstrated to be a powerful method in molecular virology, and a promising new therapeutic tool to inhibit virus replication and prevent virus-induced pathogenesis (Andino, 2003; Colbère-Garapin et al., 2005; van Rij and Andino, 2006). Recent studies associated with human and animal viruses have shown that RNAi induced by small interfering RNAs (siRNAs) is able to effectively inhibit viral replication (Huelsmann et al., 2006; Wu et al., 2005; de los Santos et al., 2005; Liu et al., 2006; H. Zhou et al., 2007; J.F. Zhou et al., 2007; Ying et al., 2007).

EMCV is one of important pathogens for swine production. The genome of EMCV is composed of a single-stranded, positive-sense RNA containing a unique large open reading frame (ORF) (Palmenberg et al., 1984), hence it may be expected that siRNA duplexes targeting any region of the genomic RNA will affect the transcription and translation of the full-length mRNA. Although a set of guidelines for the choices of potential siRNA duplexes have been proposed (Ui-Tei et al., 2004; Reynolds et al., 2004; Boese et al., 2005; Santoyo et al., 2005), there are no reliable methods for selecting the effective target regions without practical test. It is well known that different siRNA duplexes induce

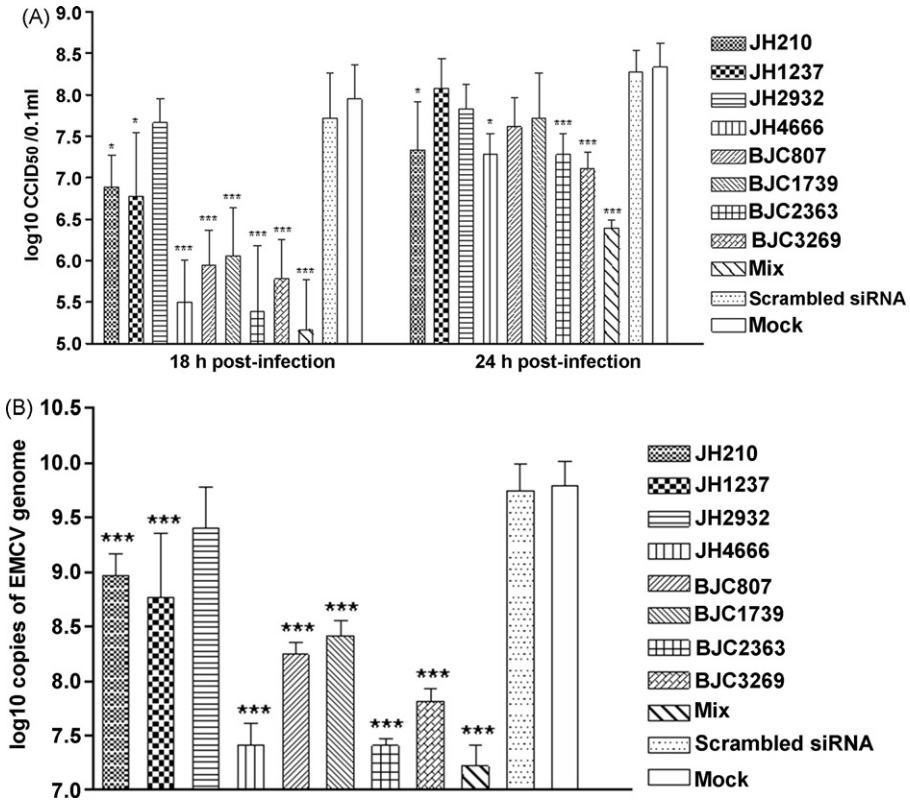

Fig. 2. Inhibitory effect of EMCV replication by the siRNAs. BHK-21 cells were transfected with 2 μg of individual siRNAs. The cells were infected with 500 $CCID_{50}$ of EMCV BJC3 at 4 h post-transfection and supernatant samples were collected for viral titration and cell lysates were collected for real-time quantitative PCR analysis. All the data were obtained by the experiments performed in triplicate and repeated three times. Error bars represent standard deviation. (A) Virus titers of supernatant samples collected at 18 and 24 h post-infection were determined as $CCID_{50}$. (B) The mRNA level of EMCV BJC3 in the transfected cells at 18 h post-infection was analyzed by real-time quantitative PCR. The data represent the copies of mRNA of 3D gene and were normalized for loading differences using mRNA of β-actin gene. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

different levels of RNAi activity (Hohjoh, 2002; Holen et al., 2002). Thus, it is important to screen appropriate target regions conferring a strong RNAi activity within the EMCV genome. Therefore, we selected eight siRNA duplexes directed to viral capsid protein 1A and 1C, viral protease 2A and 3C, viral nonstructural protein 2B and 2C, and RNA-dependent RNA polymerase 3D of EMCV BJC3 genome by Ambion's siRNA "Target Finder and Design Tool" available at http://www.ambion.com/techlib/misc/siRNA_finder. The ability of these siRNAs to inhibit EMCV replication in BHK-21 cells was assessed by examinations of EMCV-induced CPE, cell viability, virus titer, mRNA expression level of 3D gene and VP1 protein expression in the transfected cells with each of the siRNA duplexes. EMCV-induced CPE examinations showed that five synthetic siRNA out of eight candidates, namely JH-4666 and BJC-1739 to 2C, BJC-807 to 2B, BJC-2363 to 3C and BJC-3269 to 3D, could remarkably inhibit EMCV replication in BHK-21 cells and delayed the appearance of CPE. Particularly, the cells transfected with JH-4666, JH-2363 and JH-3269 displayed CPE typical of EMCV until 24 h post-infection. The CPE differences among the treated cells were apparently observed at 18 h post-infection. Thus, we selected 18 h post-infection as the appropriate time point for the subsequent assay. Viral titration analysis indicated that the virus titer of cell culture supernatants treated with JH-4666, BJC-807, BJC-1739, BJC-2363 and BJC-3269 was reduced by approximately 166-, 46.5-, 40-, 215-, 87-fold at 18 h post-infection, respectively, compared to scrambled siRNA duplex treatment. BJC-2363, targeting the 3C gene, appeared to be the most effective inhibitor of EMCV replication, followed by JH-4666 and BJC-3269. The other synthetic siRNAs (JH-210, JH-1237 and JH-2932) showed no inhibitory effects on EMCV replication. Probably, the target sequences of JH-4666, BJC-807, BJC-1739, BJC-2363 and BJC-3269 are highly conserved (Kassimi et al., 2002; G.Q. Zhang et al., 2007).

The target 3C viral protease gene, 3D RNA-dependent RNA polymerase gene, and 2C ATPase genes are necessary for the EMCV life cycle (Morasco et al., 2003; Teterina et al., 2006). In the presence of 3C, 3D and 2C gene-specific siRNAs, the life cycle of EMCV was affected and the newly transcribed mRNA was degraded, resulting in inhibition of 3C, 3D and 2C protein synthesis. Thus, we speculated that further polyprotein processing and RNA replication of EMCV were inhibited from the results of the virus titration assay, real-time PCR and Western blotting analysis as well as the decreased quantity of the progeny virus (Figs. 2–4). In addition, the results obtained using PRRSV as virus control (Fig. 5) showed that the inhibition of EMCV replication mediated by the designed siRNAs was specific to target mRNA, and not a global down-regulation resulting from activation of the double stranded RNA (dsRNA)-activated protein kinase R, inducing a non-specific,

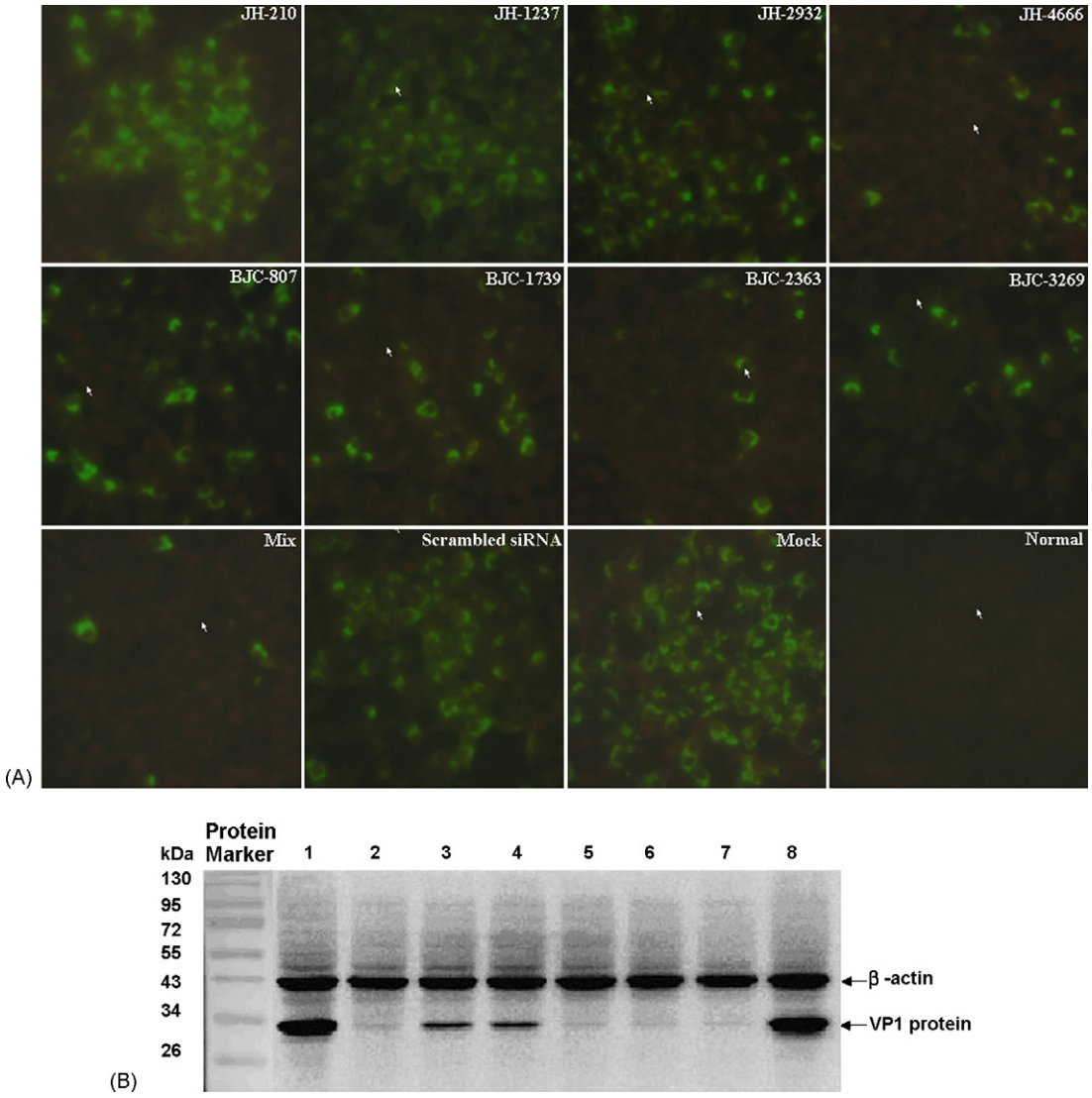

Fig. 3. Analysis of inhibition effect of EMCV BJC3 VP1 expression by the siRNAs. (A) The cells transfected with individual siRNAs, mock control and normal cells were fixed at 12 h post-infection and were used for IFA using a monoclonal antibody against EMCV VP1 protein (C11). (B) Western blotting analysis of EMCV VP1 expression of the transfected cells. Supernatant samples of the transfected cell lysates collected at 18 h post-infection were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred to nitrocellulose membrane. Lanes 1–8 showed the staining protein band of the cells transfected with the siRNAs JH-2932, JH-4666, BJC-807, BJC-1739, BJC-2363, BJC-3269, mixture of siRNAs and scrambled siRNA, respectively.

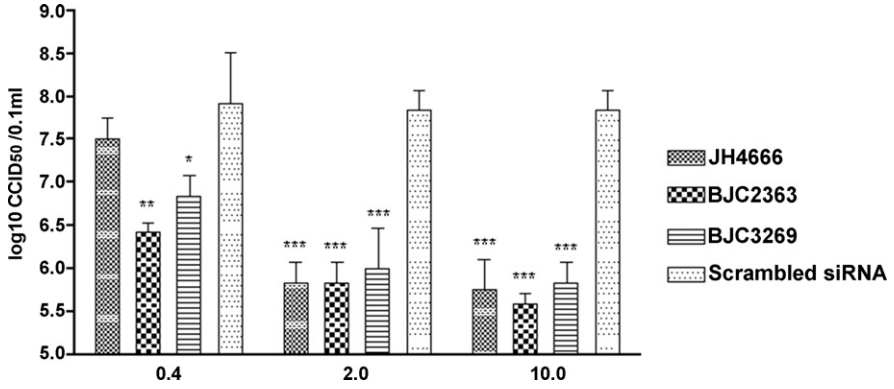

Fig. 4. Dose-dependent inhibition of EMCV replication on BHK-21 cells by the siRNAs. BHK-21 cells were transfected with 0.4, 2 and 10 μg of siRNAs JH-4666, BJC-2363 and BJC-3269, respectively, and followed by infection with 500 $CCID_{50}$ of EMCV BJC3. Supernatant samples were collected at 18 h post-infection. Virus titers were determined from two independent experiments, and the data represent the mean ± S.D. of two independent experiments in triplicate. Error bars represent standard deviation.*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

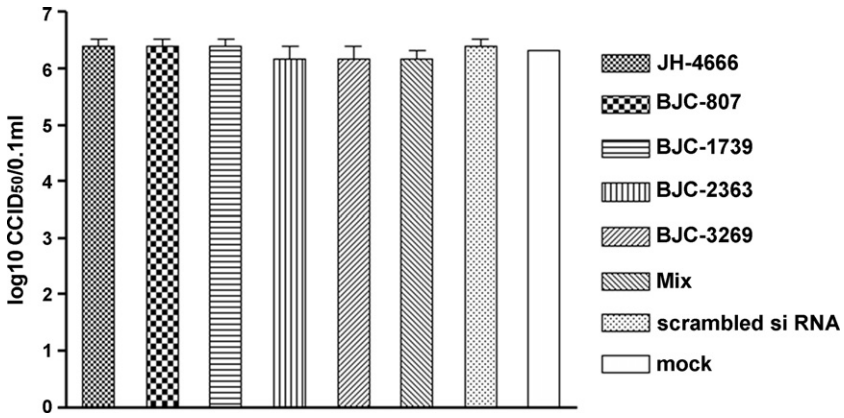

Fig. 5. No off-target inhibition of PRRSV replication in MARC-145 cells by the siRNAs. MARC-145 cells were transfected with 2 μg of JH-4666, BJC-807, BJC-1739, BJC-2363, BJC-3269, mixtures of siRNAs, or scrambled siRNAs. The cells were infected with 500 $CCID_{50}$ of PRRSV BJ-4 at 4 h post-transfection and the supernatants were collected for viral titration after twice freeze-thawing. Virus titers of supernatant samples collected at 48 h post-infection were determined as $CCID_{50}$. All the data were obtained from two independent experiments in triplicate. Error bars represent standard deviation.

interferon-mediated general antiviral response. In summary the siRNA duplexes shown could effectively trigger the degradation of EMCV genome RNA and exert a potent inhibition of EMCV replication in BHK-21 cells.

A number of hypotheses about the gene-silencing efficiency determination have been proposed, including local protein factors binding on the mRNAs (Holen et al., 2002) and the local higher structure of the targeted mRNAs (Bohula et al., 2003; Kretschmer-Kazemi Far and Sczakiel, 2003; Luo and Chang, 2004; Vickers et al., 2003). To further make clear the mechanism of determining gene-silencing efficiency, considerable work needs to be done. Although our present data have proved that the siRNAs (JH-4666, BJC-2363 and BJC-3269) are capable of effectively inhibiting EMCV replication in vitro, the difficulty we face is how to directly apply them in animals as a novel chemotherapic entity in the future. Firstly, the siRNAs are negatively charged and do not readily cross cell membrane. An effective siRNA-mediated prevention and treatment of viral infection require efficient means to deliver siRNAs into target cells (H. Zhou et al., 2007; J.F. Zhou et al., 2007). Polyethylenimines (PEIs) and liposomes/cationic lipids have been used for the systemic or local application of siRNAs in vivo (Aigner, 2006). Secondly, the results in our study showed that the inhibition of chemically synthesized siRNA is transient, maybe they are readily degraded enzymatically and nonenzymatically. One approach to solve this problem is the use of DNA expression plasmids or viral vectors which encode palindromic hairpin loops with the desired sequence (Aigner, 2006). Finally, chemically synthesized siRNA will be too expensive to use in pigs, humans or xenotransplantation. Therefore, in the future we need to balance the cost benefit ratio of using siRNAs as a real therapy reagent, and to utilize DNA expression plasmids or viral vectors for delivering the efficient siRNAs (JH-4666, BJC-2363 and BJC-3269) in vivo either systemically or locally in piglets.

Some evidence has revealed that EMCV infection has potential significance in public health worldwide (Deutz et al., 2003a,b; Juncker-Voss et al., 2004; Kirkland et al., 1989). Thus, it is necessary to prevent EMCV infection in pigs not only for swine production, but also for future xenotransplantation in human and treatment of EMCV infection in human. Determination of the effective siRNAs for inhibition of EMCV replication in vitro makes the possibility for developing an antiviral therapy approach and combining with medicinal-chemical products or vaccines to treat the early infection of EMCV in piglets. To our knowledge, this is the first report to show the effectiveness of RNA interference for inhibition of EMCV replication in vitro. In conclusion, our results indicate that chemically synthesized siRNAs duplexes targeting the 2C, 2B, 3C and 3D genes of EMCV genome could rapidly and specifically inhibit EMCV replication in BHK-21 cells in early stage of infection. Our data provide valuable evidence for further investigation of efficient RNAi technology against EMCV infection using animal models.

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