



Significant inhibition of re-emerged and emerging swine enteric coronavirus *in vitro* using the multiple shRNA expression vector

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ABSTRACT

Swine enteric coronaviruses (SECoVs), including porcine epidemic diarrhea virus (PEDV), swine acute diarrhea syndrome coronavirus (SADS-CoV), and porcine deltacoronavirus (PDCoV) have emerged and been prevalent in pig populations in China for the last several years. However, current traditional inactivated and attenuated PEDV vaccines are of limited efficacy against circulating PEDV variants, and there are no commercial vaccines for prevention of PDCoV and SADS-CoV. RNA interference (RNAi) is a powerful tool in therapeutic applications to inhibit viral replication *in vitro*. In this study, we developed a small interfering RNA generation system that expressed two different short hairpin RNAs (shRNAs) targeting the M gene of PEDV and SADS-CoV and the N gene of PDCoV, respectively. Our results demonstrated that simultaneous expression of these specific shRNA molecules inhibited expression of PEDV M gene, SADS-CoV M gene, and PDCoV N gene RNA by 99.7%, 99.4%, and 98.8%, respectively, in infected cell cultures. In addition, shRNA molecules significantly restricted the expression of M and N protein, and impaired the replication of PEDV, SADS-CoV, and PDCoV simultaneously. Taken together, this shRNAs expression system not only is proved to be a novel approach for studying functions of various genes synchronously, but also developed to test aspects of a potential therapeutic option for treatment and prevention of multiple SECoV infections.

1. Introduction

Swine enteric coronaviruses (SECoVs), including porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV), have emerged and spread throughout the swine industry in China in recent years (Dong et al., 2016; Li et al., 2012; Song et al., 2015). Swine acute diarrhea syndrome coronavirus (SADS-CoV) is a novel coronavirus which was first reported in southern China in 2017. It also can cause severe diarrhea disease in newborn piglets (Fu et al., 2018; Gong et al., 2017; Pan et al., 2017; Wang et al., 2018a, 2018b; Xu et al., 2018; Zhou et al., 2018a, 2018b). Similar to other SECoVs, such as transmissible gastroenteritis virus (TGEV), these re-emerged and emerging SECoV diseases are age-dependent with high morbidity and mortality in neonatal pigs. Mixed infections of PEDV, SADS-CoV, and PDCoV are presented in swine herds, which mostly are double infections or successive

infections (Ajayi et al., 2018; Niederwerder and Hesse, 2018; Trudeau et al., 2017; Zhou et al., 2018b). Currently, vaccination is the primary measure for prevention of PEDV. However, genetic variants of PEDV have been identified in Asia, Europe, and North America since 2010 (Bevins et al., 2018; Guo et al., 2018; Su et al., 2018; Yu et al., 2018). Traditional inactivated and attenuated PEDV vaccines failed to provide robust protection against PEDV variants infection (Lee et al., 2018; Li et al., 2017, 2018b; Park and Shin, 2018). Meanwhile, SADS-CoV and PDCoV have no commercial vaccines yet (Fu et al., 2018; Hu et al., 2015; Ma et al., 2015). Therefore, given complexity of mixed viral infection and issues associated with novel antigenic variants, novel antiviral strategies may provide means of effectively addressing issues of SECoV infection in swine.

RNA interference (RNAi) is a process in which the gene expression can be silenced in a sequence-specific manner mediated by shRNA,

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Table 1
The inserted sequences in shRNA-expressing plasmids.

Name	Sequence ^a
<i>BglIII</i> -PEDV/SADS-M- <i>EcoRI</i> -F:	5'-gatcGCTGGAATWTCAYAYGGAATtcaagagaATTCCRTRTGAWATTCCAGTTTTTTg-3'
<i>BglIII</i> -PEDV/SADS-M- <i>EcoRI</i> -R:	5'-aattcAAAAAACTGGAATWTCAYAYGGAATtctctttaaATTCCRTRTGAWATTCCAGC-3'
<i>XhoI</i> -PDCoV-N- <i>KpnI</i> -F:	5'-tcgagGGCTGACACTTCTATTTAAAtcaagagaTTTAATAGAAGTGCAGCCTTTTTTggtac-3'
<i>XhoI</i> -PDCoV-N- <i>KpnI</i> -R:	5'-cAAAAAAGGCTGACACTTCTATTTAAAtctctttaaTTTAATAGAAGTGCAGCCc-3'
<i>BglIII</i> -NC- <i>EcoRI</i> -F:	5'-gatcGCTAGTATGTGCGTGGCGTTtcaagagaAACCGCACGCACATACTAGTTTTTTg-3'
<i>BglIII</i> -NC- <i>EcoRI</i> -R:	5'-aattcAAAAAACTAGTATGTGCGTGGCGTTtctctttaaAACCGCACGCACATACTAGC-3'
<i>XhoI</i> -NC- <i>KpnI</i> -F:	5'-tcgagCTAGTATGTGCGTGGCGTTtcaagagaAACCGCACGCACATACTAGTTTTTTggtac-3'
<i>XhoI</i> -NC- <i>KpnI</i> -R:	5'-cAAAAAACTAGTATGTGCGTGGCGTTtctctttaaAACCGCACGCACATACTAGc-3'

^a Lowercase letters indicated the end sequences of the enzyme digestion reaction; Underlined lowercase letters indicated the loop sequences; Uppercase letters indicated the siRNA sequences. W: A or T; Y: C or T; R: A or G.

making it a powerful approach in therapeutic applications (Berkhout, 2018). In recent years, RNAi has been successfully developed as a new antiviral therapy regimen, and widely used to inhibit viral replication *in vitro* and *in vivo*, including against human immunodeficiency virus type 1 (HIV-1) (Lau et al., 2007), severe acute respiratory syndrome coronavirus (Li et al., 2005), influenza virus (Sui et al., 2009), classical swine fever virus (Li et al., 2011), foot-and-mouth disease virus (Oh et al., 2018) and TGEV (Zhou et al., 2010). A plasmid-transcribed shRNA against PEDV has been reported (Shen et al., 2015). However, the single-shRNA expression vector has several limitations in protecting from SECoV infection, especially under the complex co-infection of multiple viruses or different genotypes. Previous studies showed that after long-term culturing, some viruses including HIV and hepatitis C virus could evolve to escape recognition of RNAi machinery by accumulating point mutations that change the target site of sequence directly or the local secondary structure nearby (Lau et al., 2007; Watanabe et al., 2014). An alternative approach for preventing this emergence of escape mutants is the simultaneous use of multiple shRNAs (Pulloor et al., 2014). Against hepatitis B virus (HBV), a plasmid encoding three shRNAs driven by a single RNA polymerase III promoter U6 was more effective in limiting viral infection than single shRNA (Chen and Mahato, 2008). In coronavirus, membrane (M) and nucleocapsid (N) genes are highly conserved among genotypes and have pivotal roles in the viral life cycle (Kuo et al., 2016; Mason et al., 2003; Stadejek et al., 2013), thus making them candidates for the design of antiviral RNAi.

For the development of a durable gene therapy that blocks the replication of multiple SECOVs simultaneously, different shRNAs targeting PEDV (CH/JXJA/2017, accession no. MF375374.1) (Li et al., 2018a), SADS-CoV (CH/FJWT/2018, accession no. MH615810.1), and PDCoV (CH/JXNI/02/2015, accession no. KR131621.1) (Song et al., 2015) were independently screened, then a single plasmid expressing multiple shRNA (multi-shRNA) against these viruses was constructed. This multi-shRNA expression system was capable of protecting against viral infection *in vitro* by silencing the sequences of PEDV, SADS-CoV, and PDCoV. Our results highlighted the feasibility of RNAi-based technology as a potential therapy against multiple SECOVs.

2. Materials and methods

2.1. Cell culture and virus propagation

Both Vero-81 cells and LLC-PK1 cells were maintained in DMEM (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO₂. These SECOVs (CH/JXJA/2017, CH/FJWT/2018, and CH/JXNI/02/2015) were isolated from sick piglets with typical symptoms of diarrhea and propagated in Vero-81 cells or LLC-PK1 cells as previously described and stored in our lab (Li et al., 2018a; Song et al., 2015; Zhou et al., 2018b).

2.2. SiRNAs sequences selection and multiple shRNAs expression plasmid construction

Two siRNA sequences for the inhibition of CH/JXJA/2017, CH/FJWT/2018, and CH/JXNI/02/2015 were designed by Block-iT™ RNAi Designer program (<http://rnaidesigner.thermofisher.com/rnaidesigner/>), and a BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>) was performed to remove all possible homologous sequences (Supplementary Table 1). Next, a shRNA expression plasmid was constructed through a series of cloning steps (Supplementary Fig. 1). Using pSpCas9(BB)-2A-mCherry as a donor, *MluI* and *BglIII* restriction endonuclease sites were added upstream and downstream, respectively, of the human U6 small nuclear promoter (hU6) sequence. Meanwhile, *HindIII* and *XbaI* restriction endonuclease sites were linked to both ends, separately, of the mCherry fluorescent protein reporter sequence gene using PCR amplification. Afterward, a second U6 promoter construct was flanked *BglIII-EcoRI* and *XhoI-KpnI-BamHI* restriction endonuclease sequences by PCR, respectively. This second U6 promoter was cloned using *BglIII/BamHI* into the *BglIII* site of to generate the multi-shRNA expression construct pSil-Double-U6-mCherry (Supplementary Fig. 1). The fragment of shRNA targeting PEDV and SADS-CoV M gene, which is identical between PEDV and SADS-CoV (Supplementary Table 1), was digested by *BglIII* and *EcoRI*, and the fragment of shRNA targeting PDCoV N gene was digested by *XhoI* and *KpnI* and then were successively cloned into pSil-Double-U6-mCherry to yield pMulti-shRNA-M/N-mCherry (Table 1). A shRNA vector pSil-shRNA-NC-mCherry targeting a non-specific sequence was generated as a nonspecific transfection control. The plasmids constructed above were validated by PCR identification and DNA sequencing.

2.3. Generation of Vero-81 or LLC-PK1 cells with stable expression of multiple-shRNA and virus infection

Vero-81 or LLC-PK1 cells were seeded (2.5×10^4 /well) into 6-well plates. When cells reached 80–90% confluence, cells were transfected with 2.5 μg/well of pMulti-shRNA-M/N-mCherry and pSil-shRNA-NC-mCherry using the Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher, USA). After incubating for 24 h, the medium was replaced with 2% FBS containing 1000 μg/ml of Neomycin (G418). These resistant cell clones were maintained in G418-containing media for 15 days with routine medium replacements until cell death could no longer be observed. Then, these Vero-81 or LLC-PK1 monoclonal cells transfected with pMulti-shRNA-M/N-mCherry and pSil-shRNA-NC-mCherry were screened by limiting dilution analysis (LDA) as described previously (Oh et al., 2018), and cultured in media with addition of G418 (500 μg/ml) in 6-well plates at 37 °C in a 5% CO₂ atmosphere. After reaching 90–100% confluence, these plasmid-transduced Vero-81 or LLC-PK1 cells were infected with PEDV, SADS-CoV, or PDCoV at 100 50% tissue culture infective dose (TCID₅₀), respectively. Untransfected Vero-81 or LLC-PK1 cells were infected by these viruses and served as a mock infection control. At 36 h post-infection (h.p.i.), cell transfection

efficiency and cytopathic effect (CPE) images were captured under an inverted fluorescence/phase-contrast microscopy (ZEISS, Germany).

2.4. Immunofluorescence staining

Immunofluorescence assays were performed to detect PEDV, SADS-CoV, and PDCoV in the established stable Vero-81 or LLC-PK1 cell lines with multi-shRNA and shRNA-NC expression as previously described (Li et al., 2018a; Song et al., 2015; Zhou et al., 2018b) using mouse monoclonal antibody against the M protein of PEDV and SADS-CoV, or the N protein of PDCoV prepared in our laboratory. After incubation for 1 h at 37 °C, cells were washed with PBS (0.01 M, pH 7.4) three times and incubated with FITC-conjugated goat anti-mouse secondary antibodies (Transgen Biotech, China) for 1 h at 37 °C. Cells were then washed with PBS and incubated in 0.1 µg/ml 4', 6-diamidino-2-phenylindole (DAPI) (Sigma, USA) for 5 min. The fluorescent images were captured using fluorescent microscopy.

2.5. MTS assay

The established stable cell lines of Vero-81 or LLC-PK1 expressing multi-shRNA and shRNA-NC were seeded into 96-well plates at a density of 1×10^4 /well. After reaching 100% confluence, the cells were challenged with PEDV, SADS-CoV, or PDCoV at 100 TCID₅₀ per well. At 24 h.p.i., cells were washed with PBS three times, and then cell viability was assessed by adding 20 µl per well of CellTiter 96 AQueous™ One Solution Cell Proliferation Assay (MTS, Promega, USA) directly into the cell culture media. The cells were incubated for 4 h with MTS reagent, and the light absorbance at 490 nm was measured. Percent cell viability (%) was calculated using the mean OD value of the treated cells relative to that of the mock infection control. The experiment performed utilizing three technical replicates.

2.6. Viral titer assay

PEDV, SADS-CoV, or PDCoV infected Vero-81 or LLC-PK1 cells with multi-shRNA and shRNA-NC expression and supernatants were collected at 48 h.p.i. After three freeze-thaw cycles and clarification by centrifugation at 4000 ×g for 15 min at 4 °C, the viral titer was measured using standard TCID₅₀ assay. The cultures were serially diluted tenfold from 10⁻¹ to 10⁻¹⁰, and added onto a monolayer of Vero-81 cells or LLC-PK1 cells in 96-well culture plates. Each dilution was added to eight wells. After 3 days of infection, CPE was assessed microscopically and TCID₅₀ was calculated by the Reed-Muench method (Li et al., 2011).

2.7. Quantitative real-time PCR

The established stable cell lines of Vero-81 or LLC-PK1 expressing multi-shRNA and shRNA-NC were harvested at 48 h.p.i. and total RNA was extracted using MiniBEST™ Universal RNA Extraction Kit (Takara, China). The mRNA levels of the targeting genes (the M gene of PEDV and SADS-CoV, and the N gene of PDCoV) and non-targeting genes (the ORF1 and S genes of PEDV, SADS-CoV, and PDCoV) were determined by quantitative real-time PCR (qPCR) using the one Step SYBR PrimeScript™ PLUS RT-PCR Kit (Takara, China) and the gene-specific primers described previously are presented in Supplementary Table 2 (Li et al., 2018a; Song et al., 2015; Zhou et al., 2010, 2018a). PCR amplification was carried out using an ABI 7500 Real-Time PCR System (Thermo Fisher, USA) under the following conditions: initial denaturation at 95 °C for 30 s, and then 40 cycles of 95 °C for 5 s and 61 °C for 30 s; the melting curve stage comprised 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. The relative amounts of viral RNAs in each sample were normalized to the expression of β-actin as an internal control by 2^{-△△Ct} method (Shen et al., 2015). All experiments were repeated three times.

2.8. Western blot

At 48 h.p.i., total proteins were extracted from cells and western blots were performed as reported previously (Fang et al., 2017; Li et al., 2015; Zhou et al., 2018b) using primary antibodies against PEDV, SADS-CoV, and PDCoV as described in the “Immunofluorescence staining” section above and a mouse polyclonal anti-β-actin for loading control (Transgen, China). Equal amount of each sample was heated at 70 °C for 10 min, separated in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then electrotransferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies at 4 °C overnight, washed three times with PBS plus 0.05% Tween-20, and incubated with horseradish peroxidase-conjugated anti-mouse IgG at 37 °C for 1 h. Proteins were detected by adding electrochemiluminescence substrate (GenScript, USA). Image analysis was performed with Image Lab software (Biorad, USA).

2.9. Statistical analysis

Statistical analysis of the data from qPCR and MTS assays were performed using GraphPad Prism Software version 5.01 (GraphPad Software, USA). Significant differences were determined by Student's *t*-test. A *p*-value of < 0.05 (*) was considered statistically significant and a *p*-value of < 0.001 (***) was considered statistically highly significant. Data were presented as mean ± standard deviation (SD).

3. Results

3.1. Sequence-specific protection of Vero-81 cells and LLC-PK1 cells from PEDV, SADS-CoV, or PDCoV-induced CPE by multiply-expressed shRNAs

To investigate whether multi-shRNA could protect Vero-81 cells or LLC-PK1 cells from CPE induced by PEDV, SADS-CoV, and PDCoV, pMulti-shRNA-M/N-mCherry-transfected cells were infected and assessed. pSil-shRNA-NC-mCherry was used as nonspecific transfection control. At 36 h.p.i. with 100 TCID₅₀ of PEDV, SADS-CoV, or PDCoV, analysis of CPE indicated that Vero-81 or LLC-PK1 cells in the mock or negative transfection controls became reticulated and detached from the monolayer. However, CPE was rarely observed in SECoV-infected Vero-81 or LLC-PK1 cells expressing specific multi-shRNA. Control staining for shRNA vector (mCherry), viral protein (FITC), and cell monolayer (DAPI) indicated that CPE was directly associated with loss of cell monolayer with viral replication (Figs. 1–3).

3.2. Increased viability of PEDV, SADS-CoV, and PDCoV infected cells induced by multiple-shRNA

To further study the effect of multi-shRNA on protecting cells against PEDV, SADS-CoV, or PDCoV infection, cell viability determined by a MTS assay was performed. Since there is a linear response between cell number and the absorbance readout, the absorbance value at 490 nm is directly proportional to the number of living cells in culture (Supplementary Fig. 2). Compared with nonspecific transfection (pSil-shRNA-NC-mCherry) control and mock (nontransfected plasmid) transfection control cells, specific transfection (pMulti-shRNA-M/N-mCherry) cells were highly effective in inhibiting SECoV-induced CPE at 28 h.p.i. This phenomenon indicated that the multi-shRNA-expressing plasmid could significantly protect cells from PEDV infection. The viability (mean ± SD %) of multi-shRNA-expressing cells was 95.13 ± 1.49% (Fig. 4a), which was obviously higher (*p* < 0.001) than that of the nonspecific transfection control (49.67 ± 0.43%) and the mock transfection control (44.90 ± 0.37%). Likewise, in Vero-81 cells infected with SADS-CoV at 28 h.p.i., these similar results were observed, indicating that the multi-shRNA-expressing plasmid could prevent cells from SADS-CoV infection. The cell viability of pMulti-shRNA-M/N-mCherry transfected cells was 97.45 ± 1.06% (Fig. 4b),

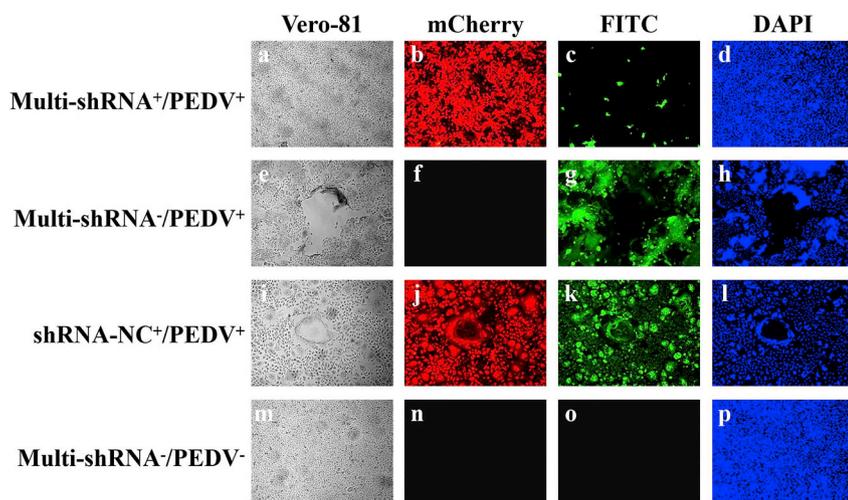


Fig. 1. Effect of multi-shRNA on PEDV-induced CPE in Vero-81 cells by immunofluorescence assay. (a–d) pMulti-shRNA-M/N-mCherry transfected cells showed remarkable reduction of viral protein expression; (e–h) Non-transfected cells with infection were used as mock transfection control; (i–l) pSil-shRNA-NC-mCherry transfected cells showed no detectable effect on virus infection as nonspecific transfection control; (m–p) Non-transfected and non-infected cells were used as untreated blank control. The bright green color indicated the presence of viral protein-positive cells. The cherry red color indicated the presence of the transfected cells. The nuclei were stained blue by DAPI ($\times 100$ magnification).

which was far greater ($p < 0.001$) than that of the nonspecific transfection control ($24.00 \pm 0.33\%$) and the mock transfection control ($24.82 \pm 0.54\%$). As expected, the similar situation in LLC-PK1 cells infected with PDCoV were observed compared to the experimental data from Vero-81 cells. It was suggested the multi-shRNA-expressing plasmid could sufficiently protect cells from PDCoV infection. In addition, the viability of cells expressing multi-shRNA was $92.87 \pm 1.6\%$ (Fig. 4c), which was considerably higher ($p < 0.001$) than that of the nonspecific transfection control ($58.03 \pm 1.39\%$) and the mock transfection control ($50.66 \pm 3.31\%$). Taken together, our results indicated that multi-shRNA-treated cell conditions maintained a level of cell viability comparable to those of uninfected controls, while SECoV-infected nonspecific-shRNA-treated cells experienced a loss of viability comparable to infected cells lacking specific shRNA expression.

3.3. Multiple-shRNA-mediated reduction in production of infectious virus

To substantiate the inhibitory effect of shRNAs on production of viable virus, $TCID_{50}$ was used to titrate PEDV, SADS-CoV, and PDCoV at 48 h.p.i. PEDV results showed that in nonspecific-transfected control cells, virus titer reached $10^{6.04 \pm 0.07} TCID_{50}/ml$, which was similar to that observed in mock transfected control cells ($10^{6.61 \pm 0.14} TCID_{50}/ml$). In contrast, the titer determined in cells transfected with pMulti-shRNA-M/N-mCherry was $10^{3.21 \pm 0.11} TCID_{50}/ml$ (Fig. 5a), which was reduced by 2500-fold ($p < 0.001$) compared to that of PEDV in mock transfected control cells. SADS-CoV data showed that the virus titers of

nonspecific transfection and mock transfection controls were $10^{5.63 \pm 0.05} TCID_{50}/ml$ and $10^{5.71 \pm 0.09} TCID_{50}/ml$, respectively. Conversely, the titer in cells transfected with pMulti-shRNA-M/N-mCherry was only $10^{2.38 \pm 0.04} TCID_{50}/ml$ (Fig. 5b) and reduced by 2100-fold ($p < 0.001$) compared to that of SADS-CoV in mock transfected control cells. PDCoV data demonstrated the virus titers in nonspecific transfection and mock transfection controls were as high as $10^{8.23 \pm 0.06} TCID_{50}/ml$ and $10^{8.53 \pm 0.08} TCID_{50}/ml$, respectively. In contrast, the titer in multi-shRNA-expressing cells was $10^{4.13 \pm 0.07} TCID_{50}/ml$ (Fig. 5c), which resulted in up to 25000-fold ($p < 0.001$) drop compared to that of PDCoV in mock transfected control cells. These data indicated that pMulti-shRNA-M/N-mCherry could significantly inhibit the complete replication *in vitro* of infectious PEDV, SADS-CoV, and PDCoV.

3.4. Multiple-shRNA-mediated inhibition of viral RNA replication

To assess the influence of multi-shRNA expression on SECOVs genomic RNA replication, the M gene of PEDV and SADS-CoV, and the N gene of PDCoV (these targeting genes of multi-shRNA expression system) were used, respectively, as proxies. A real-time RT-PCR quantitative analysis of mRNA expression level of M or N was normalized to the corresponding β -actin in the same sample. The relative amounts of M or N gene in SECoV-infected transfected control cells were regarded as 1.000 (Shen et al., 2015). In PEDV, the relative expression levels of the M gene in multi-shRNA or shRNA-NC expressing cells were

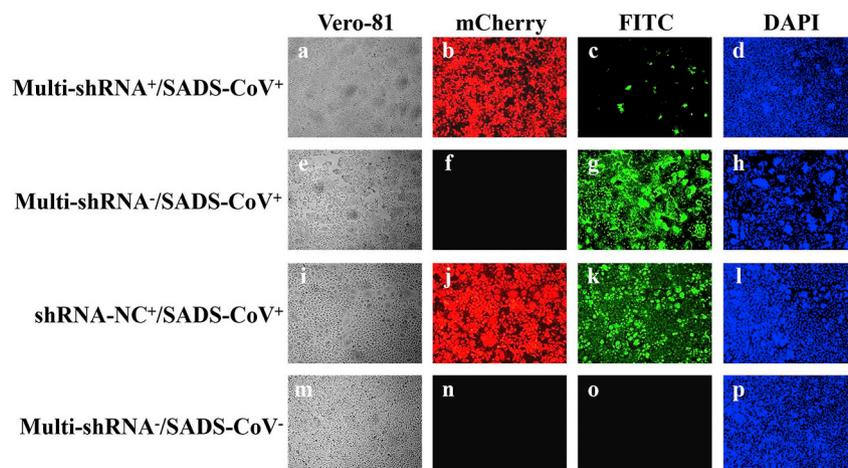


Fig. 2. Effect of multi-shRNA on SADS-CoV-induced CPE in Vero-81 cells by immunofluorescence assay. Treatments of cells and illustrations were the same as those described in legend to Fig. 1.

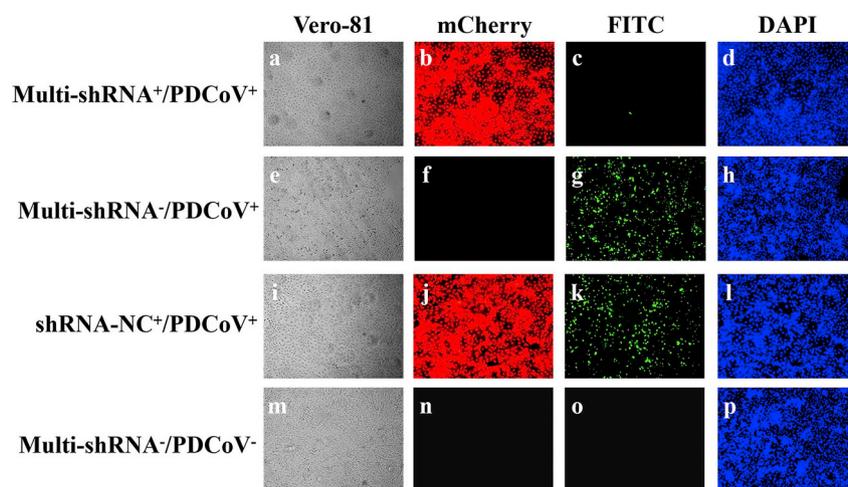


Fig. 3. Effect of multi-shRNA on PDCoV-induced CPE in LLC-PK1 cells by immunofluorescence assay. Treatments of cells and illustrations were the same as those described in legend to Fig. 1.

0.003 ± 0.001 or 0.887 ± 0.003, respectively. These data indicated that PEDV genome RNA in specific-transfected cells were reduced by 99.7%, compared to those of the mock transfection control ($p < 0.001$) (Fig. 6a). There is a similar situation in SADS-CoV infection. The relative amounts of the M gene in cells transfected with pMulti-shRNA-M/N-mCherry and pSil-shRNA-NC-mCherry were 0.006 ± 0.001 and 0.977 ± 0.002, which indicated SADS-CoV genome RNA in multi-shRNA expressing cells were dropped by 99.4%, compared to those of the non-transfection control ($p < 0.001$) (Fig. 6b). The relative amounts of the N gene in PDCoV-infected cells containing pMulti-shRNA-M/N-mCherry and pSil-shRNA-NC-mCherry were 0.012 ± 0.001 and 0.963 ± 0.004. Compared to those of the negative transfection control, the specific-transfected group showed that the amounts of PDCoV genome RNA in cells expressing multi-shRNA were reduced by 98.8% ($p < 0.001$) (Fig. 6c). The mRNA expression levels of the ORF1 and S genes, non-targeting genes of multi-shRNA expression system, of SECOVs were tested by qPCR as well, and the results were consistent with those of the targeting genes (Supplementary Fig. 3). Overall, these data suggested a potent inhibition of PEDV, SADS-CoV, and PDCoV genome RNA replication triggered by sequence-specific multi-shRNA in Vero-81 cells or LLC-PK1 cells.

3.5. Detection of the levels of viral proteins by western blot

To further investigate the effect of multi-shRNA on protein synthesis of PEDV, SADS-CoV, and PDCoV, the expression levels of viral proteins at 48 h.p.i. were examined using western blot. Fig. 7 shows that the amounts of viral proteins including PEDV N protein, SADS-CoV M protein, and PDCoV N protein harvested from the multi-shRNA expressing cells were dramatically reduced, which was consistent with the qPCR analysis (Fig. 6 and Supplementary Fig. 3) and with reduced viral protein staining in cultures expressing multi-shRNA (Figs. 1–3).

4. Discussion

SECOVs, which lead to substantial economic losses, are the major concern in swine severe diarrhea diseases since there currently are no effective treatments. RNAi technology might represent a potential strategy for the control of these diseases. Successful RNAi on the inhibition of PEDV replication has been demonstrated by synthetic siRNA and plasmid-transcribed shRNA targeting M gene (Shen et al., 2015). However, recent epidemiological studies revealed that the presence of multiple virus strains simultaneously circulated in many endemic areas including PEDV, SADS-CoV, and PDCoV (Ajayi et al., 2018;

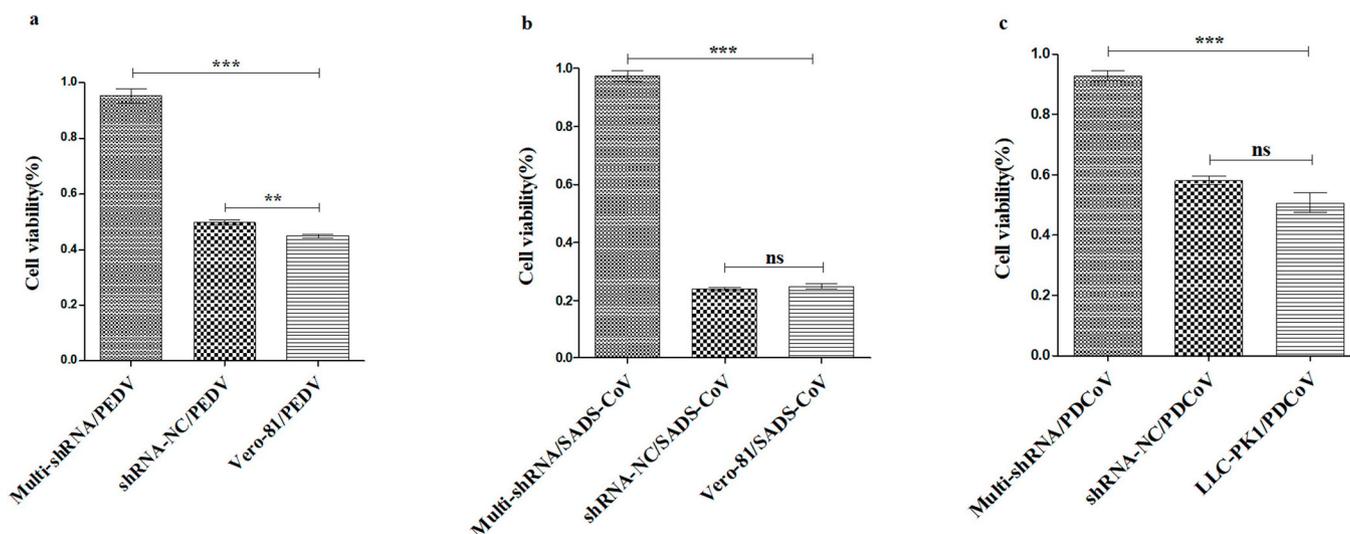


Fig. 4. The Vero-81 cells or LLC-PK1 cells viability was calculated using the MTS assay at 28 h.p.i. (a) PEDV infected group, (b) SADS-CoV infected group, (c) PDCoV infected group.

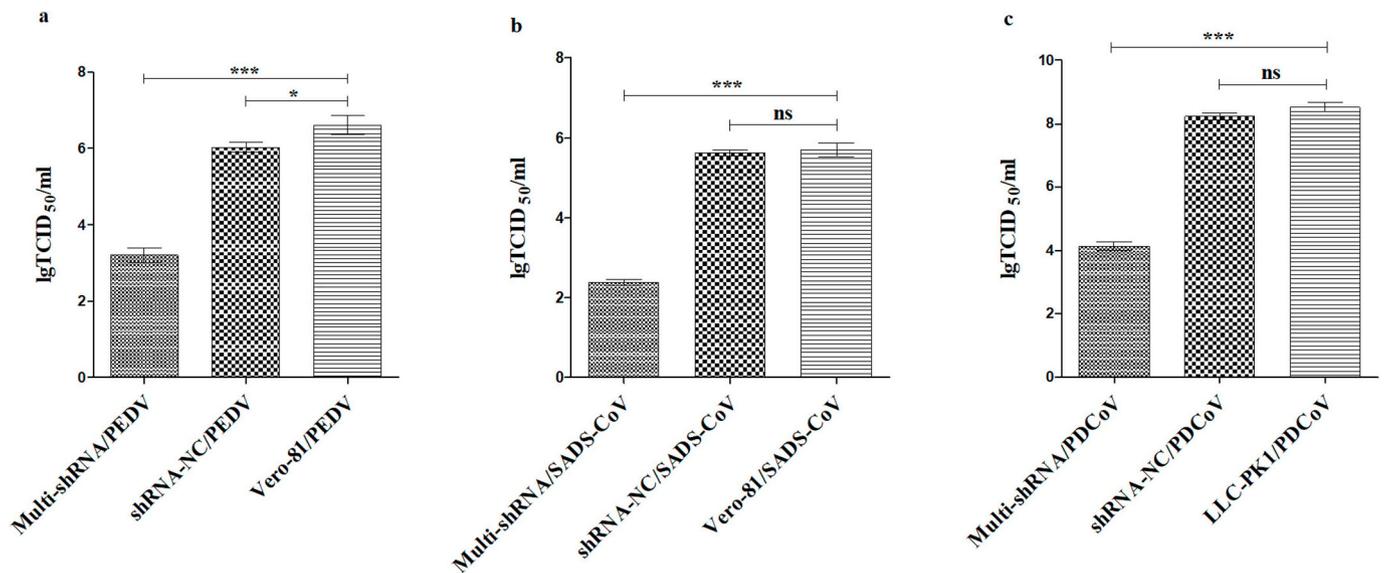


Fig. 5. Reduction in titers of PEDV (a), SADS-CoV (b), and PDCoV (c) in Vero-81 cells or LLC-PK1 cells expressing multi-shRNA.

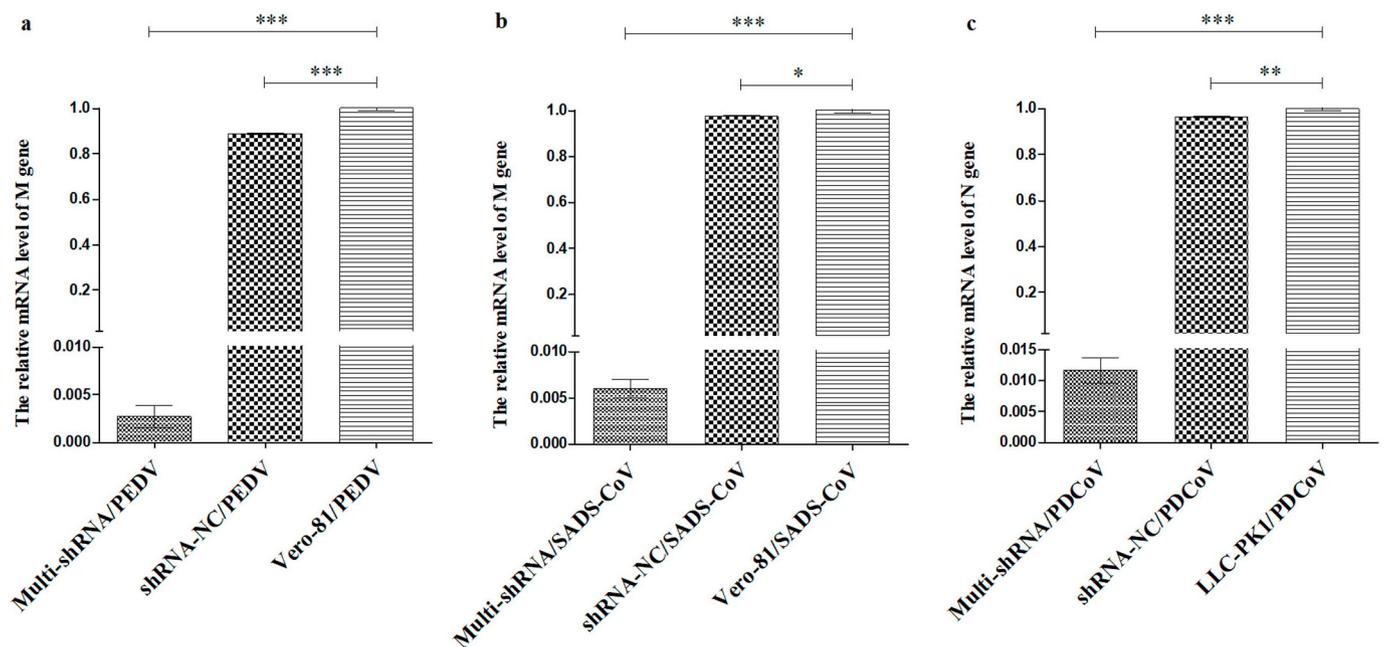


Fig. 6. Inhibition of PEDV, SADS-CoV, and PDCoV RNA replication by multi-shRNA in Vero-81 cells or LLC-PK1 cells. Quantitative real-time PCR detection of PEDV M gene (a), SADS-CoV M gene (b), and PDCoV N gene (c) mRNA transcripts relative to β -actin transcripts in the same sample.

Niederwerder and Hesse, 2018; Trudeau et al., 2017; Zhou et al., 2018b). Both PEDV and SADS-CoV are classified into group I of the genus *Alphacoronavirus*, and their membrane gene share highly similar nucleotide structure. The M protein of coronavirus not only plays a vital role in the viral assembly, but also can neutralize anti-M antibody under the existence of complement (Raaben et al., 2009). Meanwhile, the N protein forms complexes with genomic RNA of coronavirus and enhances the process of virus transcription and assembly. The N protein is the predominant antigen produced in infected cells, which makes it a major target for the accurate and early diagnosis (Kuo et al., 2016). Therefore, it is essential to design a dual-shRNA system that expresses multiple shRNAs to block a variety of SECoVs simultaneously.

For the development of a durable gene therapy that prevents infection from a range of SECoVs, an expression vector was designed to simultaneously express multiple shRNAs targeting the M protein gene of PEDV and SADS-CoV, and the N protein gene of PDCoV. The shRNA

expression cassettes in this vector were designed with a *Bam*HI restriction site compatible with *Bgl*II and allowing combination of two or more shRNAs expression cassettes in a building block manner. An analogous cloning strategy has been independently applied in the construction of a vector that was intended to silence two endogenously expressed genes simultaneously. The advantages of multi-shRNA expression vector is exhibited using a model system simulating the emergence of mutated viruses (Ma et al., 2014). This approach also could provide a possible therapeutic option against multiple-virus coinfections.

In order to express multiple shRNAs, we utilize a dual promoter system containing two copies of the hU6 promoter, an RNA Pol III promoter. Pol III promoters have been widely used to mediate the high-level expression of shRNAs. However, shRNA expression driven by RNA Pol III promoters has been shown to induce cell toxicity and lethality in adult mice (Giering et al., 2008). While cell toxicity potentially

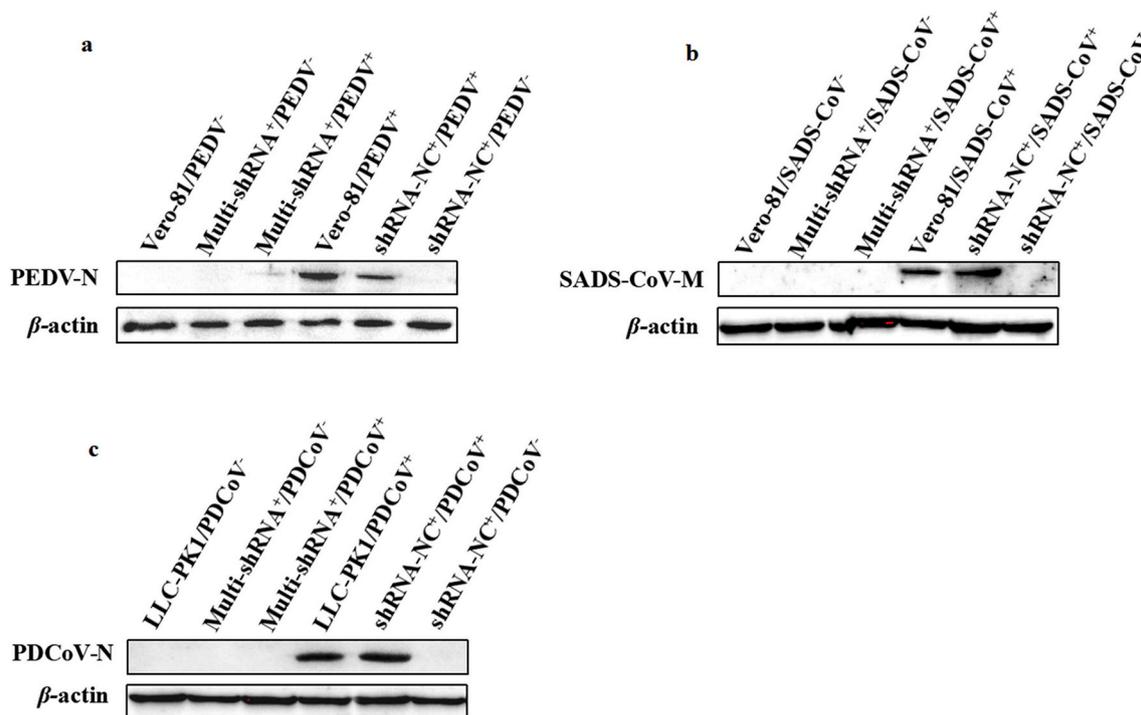


Fig. 7. Expression of viral proteins of PEDV (a), SADS-CoV (b), and (c) PDCoV assessed by western blot. Equal amounts of cell lysates at 48 h.p.i. were examined using monoclonal antibodies against N protein of PEDV, M protein of SADS-CoV, or N protein of PDCoV, with β -actin as a protein loading control.

associated with two hU6 promoters was a concern in this study, no significant cytotoxic effects were observed in Vero-81 or LLC-PK1 cells. Our results were consistent with the previous study of adenovirus vector expressing two shRNAs under control of two hU6 promoters, which did not show any obvious cytotoxicity in IBRS-2 cells (Kim et al., 2008). In contrast, the adenovirus with three separate hU6 promoters has some cytotoxic effects (Chen and Mahato, 2008). The increased toxicity might be associated with the interference of the cellular RNAi pathway induced Pol III promoter (Giering et al., 2008). Therefore, to ensure efficient inhibition of viral replication *in vitro*, we constructed that the multi-shRNA expression plasmid flanked two independent hU6 promoters to minimize the cytotoxicity potentially caused by excessive RNA Pol III promoter. In this report, the multi-shRNA expression system can inhibit virus replication without significant cytotoxicity *in vitro*. When the multi-shRNA expression vector was employed against the PEDV, SADS-CoV, and PDCoV simultaneously, it was found to silence the target gene with high potency, comparable to that of the individual single-shRNA expression vector (Shen et al., 2015; Zhou et al., 2010). This phenomenon also was observed in the experiment data from corresponding study on simultaneous expression of multiple shRNAs for the target genes of HIV and HBV, which had antiviral effects against both these viruses, showing a significant efficiency compared with that of separate applications of each single shRNA (Wu et al., 2007).

In conclusion, we introduced a novel multiple-resistance strategy against three major harmful viral diarrhea diseases in neonatal pigs. Notably, this work represents a significant advance, potentially facilitating new experimental approaches for the analysis of both viral and cellular gene functions in the context of infection with multiple infections with SECovs. Taken together, our data show that the tremendous potential for this multiple-shRNA expression vector is enable to precisely and effectively interfere with the replication of multiple SECovs *in vitro*.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.antiviral.2019.03.010>.

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