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Antiviral Research 74 (2007) 36–42
Antiviral Research
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Effective inhibition of porcine transmissible gastroenteritis virus replication in ST cells by shRNAs targeting RNA-dependent RNA polymerase gene

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Received 22 June 2006; accepted 29 December 2006

Abstract

Transmissible gastroenteritis virus (TGEV) is identified as one of the most important pathogenic agents during swine enteric infection, leading to high mortality in neonatal pigs and severe annual economic loss in swine-producing areas. Up to date, various vaccines developed against TGEV still need to be improved. To exploit the possibility of using RNA interference (RNAi) as a strategy against TGEV infection, two shRNA-expressing plasmids (pEGFP-U6/P1 and pEGFP-U6/P2) targeting the RNA-dependent RNA polymerase (RdRp) gene of TGEV were constructed and transfected into swine testicular (ST) cells. The cytopathic effect (CPE) and MTS assays demonstrated that both shRNAs were capable of protecting cells against TGEV invasion with very high specificity and efficiency. A real-time quantitative RT-PCR further confirmed that the amounts of viral RNAs in cell cultures pre-transfected with the two plasmids were reduced by 95.2% and up to 100%, respectively. Our results suggest that RNAi might be a promising new strategy against TGEV infection.

Keywords: TGEV; RNA interference (RNAi); Short hairpin RNA (shRNA); RdRp; ST

1. Introduction

Transmissible gastroenteritis virus (TGEV), the causative agent of porcine transmissible gastroenteritis (TGE), at all ages of pigs results in gastroenteritis, characterized by vomiting, yellowish diarrhea and dehydration. The consequences of TGE vary among different ages of pigs. Suckling piglets often suffer a mortality of as high as 100% (Saif and Wesley, 1999; Kim and Chae, 2001), while older animals generally show growth retardation due to high morbidity, both of which result in enormous economic loss in swine-producing areas in the world every year (Chen and Schifferli, 2003; Schwegmann-Wessels and Herrler, 2006; Sestak et al., 1996).

As a member of the *Coronaviridae*, TGEV has a genome of positive, single-stranded RNA. The genomic RNA has a length of 28.5 kb. Approximately 5′ two-thirds of the genome (from the 5′-end) comprises open reading frames (ORF) 1a and 1b, mainly encoding the RNA-dependent RNA polymerase (RdRp), whereas the one-third from the 3′-end is mainly made up of the genes encoding viral structural proteins, such as spike (S), envelope (E), membrane (M) and nucleocapsid (N) protein (Alonso et al., 2002; Escors et al., 2003; Penzes et al., 2001). The genome itself may also serve as mRNA, together with other six subgenomic mRNAs transcribed discontinuously from the negative-strand template, forming a nested set of RNAs of different lengths with co-terminal ends (Alonso et al., 2002; Lai and Cavanagh, 1997; Laude et al., 1990).

Vaccines have been considered as the best control of TGEV infections (Ho et al., 2005; Saif, 1996; Torres et al., 1995; Wesley and Lager, 2003). However, many vaccine strategies developed so far have not been entirely successful. Firstly, inactivated TGEV vaccines generally offer poor protection in swine, while the attenuated ones are not ideal in their safety due to the risk of reverting to a virulent form, and some mucosal vaccines may even induce adverse reactions (Holmgren et al., 2003; Opriessnig et al., 2002; Saif and Wesley, 1999; Wesley and

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doi:10.1016/j.antiviral.2006.12.007

Table 1
The inserted sequences in shRNA-expressing plasmids

plasmids	inserts
pEGFP-U6/P1	5'-GTACTGGGATCGCACATATTTCAAGACGATATGTGCGATCCCAGTACTTTTTT-3'
pEGFP-U6/P2	5'-GGCAAGAGCTCGTACAGTATTCAAGACGTACTGTACGAGCTCTTGCCTTTTTT-3'
pEGFP-U6/T	5'-GACTTCATAAGGCGCATGCTTCAAGACGGCATGCGCCTTATGAAGTCTTTTTT-3'

Lager, 2003). Furthermore, neonatal piglets may suffer from gastroenteritis within 20 h post-infection and death may happen in 1–4 days (Schwegmann-Wessels and Herrler, 2006), whereas current vaccines cannot provide complete protection prior to 7 days after inoculation (Brim et al., 1995; de los Santos et al., 2005). So, it is urgently needed to develop a high-effective, rapid-acting antiviral strategy against TGEV. On the other hand, current research on antiviral protection triggered by RNA interference (RNAi) in vivo mainly employed mice as experimental models (Liu et al., 2006; Palliser et al., 2006). However, studies of candidate therapeutic strategies using an adult mouse model of subclinical enteric viral infections often do not predict their efficacy in neonatal large mammals (Yuan and Saif, 2002).

RNAi is a biological process that functions as an ancient defense mechanism of cells against hostile genes and regulates the function of normal genes during growth and development (Arenz and Schepers, 2003; Bagasra and Prilliman, 2004; Ratcliff et al., 1997; Zambon et al., 2006). Since it was found that experimental introduction of exogenous double-stranded RNA into *C. elegans* and mammalian cells could specifically silence homologous mRNA (Fire et al., 1998; Elbashir et al., 2001), RNAi has been the most fascinating technique for hostile gene knockdown and viral inhibition (Borkhardt and Heidenreich, 2004; Robson and Sacks, 2005; van Rij and Andino, 2006). Within a few years, a wide variety of disease-associated viruses have been targeted effectively throughout plant, animal and human kingdom, such as severe acute respiratory syndrome coronavirus (SARS-CoV), human immunodeficiency virus (HIV), influenza virus and hepatitis viruses (Huelsmann et al., 2006; Li et al., 2005; Shlomai and Shaul, 2004; van Rij and Andino, 2006; Wu et al., 2005). Here, we report the first study to investigate whether shRNA-mediated RNA interference could inhibit TGEV replication in swine testicular (ST) cells.

2. Materials and methods

2.1. Cell culture, virus propagation and titration

Prior to being challenged with TGEV TGEs-1 strain, ST cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% heat-inactivated super neonatal bovine serum (NBS), 5% L-glutamine and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) in a 37 °C, 5% CO_2 incubator overnight. After 70% of the virus-infected cells showed cytopathic effects (CPE), the cultures were collected for three frozen–thawed cycles, and then serially diluted by 10-fold. Each dilution was added to ST cells in four-fold. After 3 days of infection, the viral 50% cell culture infectious dose ($CCID_{50}$) was calculated using the Reed and Muench method.

2.2. ShRNA sequences selection and expression plasmids construction

The RdRp gene of TGEV is well conserved among different TGEV strains and was used as the RNAi target in this study. Lots of sequences homologous to the RdRp coding region of TGEV SC-Y strain (GenBank accession no.: DQ443743) were generated by the web site siRNA designing tools (http://www.ambion.com/techlib/misc/siRNA_finder and http://www.oligoengine.com/Home/mid_prodSirna.html_sirna_tool), from which two theoretically effective sequences at nt positions 13160–13180 (P1) and 13949–13969 (P2) within the open reading frame 1b were selected. To guarantee a similar RNAi effect on different TGEV strains, the two sequences were analyzed by a BLAST search in the GenBank nucleotide database to avoid any similar sequence found in the swine genome, but share a 100% homology within the published sequences of different TGEV strains. To investigate the specificity of viral inhibition, a non-specific sequence (T) also underwent a BLAST analysis and served as a negative control. Their corresponding sequences are separately shown in Table 1. To investigate whether shRNA-mediated RNAi could block TGEV infection in ST cells, all the three sequences were generated to make shRNA-expressing plasmids: pEGFP-U6/P1, pEGFP-U6/P2 and pEGFP-U6/T, respectively. The three inserted sequences were arranged as the following alignment: *Bam*HI+ Sense+ Loop+ Antisense+ Termination signal+ *Sal*I+ *Hin*dIII (Table 1). Enhanced green fluorescence protein (EGFP) gene fused in the plasmids was used as a reporter during the transfection efficiency analysis. The RNA transcripts from these U6 promoter-containing plasmids were expected to fold back and form a stem-loop structure with a 19-base pair region homologous to the RdRp gene of TGEV.

2.3. Cell transfection and virus infection

One day before transfection, ST cells were seeded in 48-well plates at a density of 2–3 × 10^4 cells/well without antibiotics. When the cells reached 70–80% confluency, the medium was

replaced and cells were transfected with 0.4 μg/well of shRNA-expressing plasmids using the TransFast™ transfection reagent (Promega) according to the manufacturer's recommendations. The cells were then incubated at 37 °C for 1 h, followed by being overlaid with high glucose DMEM containing 5% NBS. At 28 h later, the medium was replaced and the cells were challenged with TGEV at 200 $CCID_{50}$, and the infection was allowed to proceed for the indicated time points. Cell transfection efficiency and CPE were evaluated under an inverted fluorescence/phase-contrast microscopy (Nikon) at different time points post-infection. Cell images were captured and the cell cultures were collected for a real-time quantitative RT-PCR analysis at 40 h post-infection.

2.4. MTS assay

ST cells (1–1.5 × 10^4 per well) seeded in 96-well plates were treated as described in Section 2.3 except that the amounts of plasmids and viruses in each well were half of those in 48-well plates. At 36 h post-infection, cell viability was assessed by adding 20 μl/well of CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS, Promega) to cell cultures according to the manufacturer's instructions. After 4 h incubation along with MTS, light absorbance of each solution was measured at 492 nm. The experiment was repeated three times and each performed in triplicate.

2.5. Virus yield reduction assay

ST cells seeded in 48-well plates were separately transfected with pEGFP-U6/P2 at 0.1, 0.2, 0.3 and 0.4 μg/well; untransfected cells (0 μg/well) were served as the mock control. At 28 h after transfection, the cells were challenged with TGEV at 200 $CCID_{50}$. At 40 h post-infection, cell cultures were harvested and viral titers were measured. The transfection was repeated two times and performed in triplicate.

2.6. Total RNAs extraction, reverse transcription and real-time quantitative PCR

TGEV-infected ST cells as well as culture supernatants were collected 40 h after viral infection. Total RNAs were extracted and purified using an RNAultra kit (Tiangen, China) according to the manufacturer's instructions. A reverse transcription and real-time quantitative PCR (RT-qPCR) analysis was conducted by using a SYBR ExScript™ RT-PCR Kit (Takara) according to the manufacturer's recommendations. The following two pairs of primers: forward, 5′-GAGCAGTGCCAAGCATTACCC-3′ and reverse, 5′-GACTTCTATCTGGTCGCCATCTTC-3′ for TGEV N gene; forward, 5′-CGTCCACCGCAAATGCTTC-3′ and reverse, 5′-AACCGACTGCTGTCACCTTCAC-3′ for β-actin gene were used. The generated PCR product of virus was mapped to 27720–27828 nuleotide (nt) region within the genome of TGEV SC-Y strain. PCR reaction was performed in a DNA Engine Opticon Chromo4™ Detector (Bio-Red MJ Research). Following a denaturation step at 95 °C for 10 s, 45 cycles of amplification were performed at 95 °C for 5 s, 58 °C for 15 s, and 72 °C for 20 s. Data were analyzed according to the comparative threshold cycle (C_t) method, where the amount of RNAs in sample (TGEV) normalized to an internal control (β-actin) and relative to a calibrator (TGEV), is given by a $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen, 2001). The tests were performed in triplicate. ΔC_t values of the triplicates were averaged before proceeding with the $2^{-\Delta\Delta C_t}$ calculation.

3. Results

3.1. Sequence-specific protection of ST cells from TGEV infection by shRNAs

3.1.1. CPE analysis

To study the TGEV-induced CPE, ST cells were infected with TGEV at 200 $CCID_{50}$. At 18 h after infection, cells were then examined with a phase-contrast microscopy every 4 h. The virus-infected cells exhibited obvious morphological changes starting from the brim of wells 19–20 h post-infection, including changes from cell fusion to cell lysis and then form large bodies on the surface during time passage. About 40 h after viral infection, the whole cell monolayer began to fall into pieces and dropped off the plate (Fig. 1E). In contrast, the non-infected cells tightly stuck to the plate in good shape all the time (Fig. 1A1 and A2).

To examine whether sequence-specific shRNAs could inhibit TGEV-induced CPE, ST cells were transfected with three shRNA-expressing plasmids (pEGFP-U6/P1, pEGFP-U6/P2 and pEGFP-U6/T) separately prior to being infected with TGEV. At 40 h after viral infection, the images of cells were captured (Fig. 1). Analysis of cell morphology indicated that the negative control (pEGFP-U6/T) had no apparent inhibitory effect on TGEV-induced CPE compared with the mock control (Fig. 1D and E). In contrast, two sequence-specific shRNA-expressing plasmids, pEGFP-U6/P1 and pEGFP-U6/P2, could potently block CPE in the cell cultures (Fig. 1B1, B2, C1 and C2). In detail, ST cells transfected with pEGFP-U6/P2 were capable of keeping CPE resistance in all the three repeated wells till cultures were harvested. There were no obvious differences among three independent experiments; however, the pEGFP-U6/P1 elicited different effects on its cells. Among the three repeated wells, two of them did not show any CPE, whereas the third showed a small patch of mild/suspected "CPE" only in cell cultures in the brim (Fig. 1B2). As to its two repeated experiments, one kept no CPE in all the repeated wells, while the other showed two small patches of "CPE" similar to the above only in one of the three replicates (data not shown).

3.1.2. MTS assay

To further study the effect of shRNAs on protecting ST cells against TGEV destruction, a MTS assay was also performed. From the data shown in Fig. 2, a result similar to that above (Section 3.1.1) was found. The mean OD values of solutions in wells treated with pEGFP-U6/P1 and pEGFP-U6/P2 were 1.93 and 2.33, respectively, while those treated with pEGFP-U6/T and untransfected (mock) were 0.55 and 0.297, respectively. For the absorbance at 492 nm is directly proportional to the number

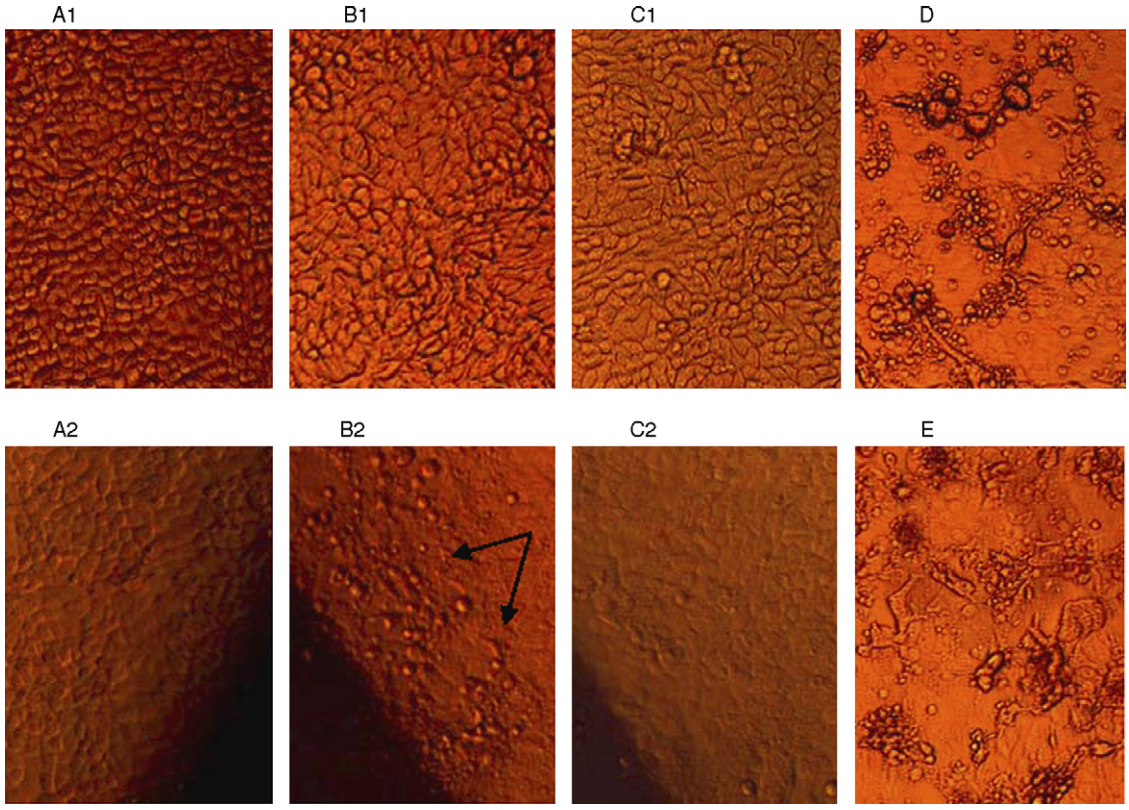

Fig. 1. Effect of shRNAs on TGEV-induced CPE in ST cells. ST cells were transfected with different plasmids and then infected with TGEV at 200 $CCID_{50}$. CPE was examined from 20 to 40 h post-infection, and cell images were captured in the end. (A1–C1) and (A2–C2) showed the images in the middle and brim of the same well, respectively. (A) ST cells neither transfected with plasmids nor infected with virus. (E) Cells only treated with virus. From (B) to (D), cells were transfected with pEGFP-U6/P1 (B), pEGFP-U6/P2 (C) and pEGFP-U6/T (D), respectively. (A, D and E) also served as a normal, negative and mock control in the experiment, respectively. The experiment was performed in triplicate and repeated three times.

of living cells in culture (examined by Promega), the number of living cells in a well treated with pEGFP-U6/P1 or pEGFP-U6/P2 was far greater than that treated with pEGFP-U6/T or mock control.

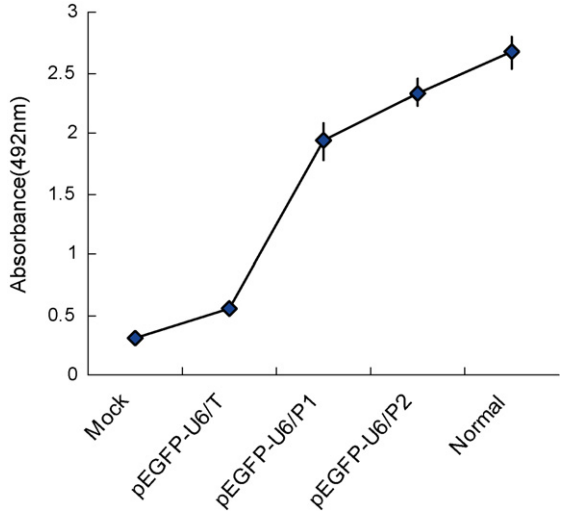

Fig. 2. Protection of ST cells from TGEV destruction by shRNAs. ST cells were transfected with plasmids as indicated and then infected with TGEV at 200 $CCID_{50}$. At 40 h post-infection including 4 h incubation along with MTS, viable cell numbers were evaluated by a MTS assay. OD values shown are the mean ± S.D. of three separate experiments performed in triplicate.

Taken together, in this study, the significant differences of CPE inhibitory effect and viable cell number between plasmids expressing sequence-specific and non-sequence-specific shRNAs demonstrated that only the two sequence-specific shRNAs could efficiently protect ST cells against TGEV infection, which suggested that the potent protections were due to sequence-specific RNA interference.

3.2. Dose-dependent inhibition of viral replication by shRNA

To optimize the inhibitory effect of shRNA on TGEV replication, a virus yield reduction assay was conducted by transfecting ST cells with pEGFP-U6/P2 at indicated doses. As shown in Fig. 3, transfection of 0.1 μg/well of pEGFP-U6/P2 just induced a slight reduction in viral titer. However, with the dose of pEGFP-U6/P2 increasing, the viral titer decreased. When the amount of pEGFP-U6/P2 reached 0.4 μg/well, the highest inhibition of TGEV replication was recorded. The data indicated that the inhibition of TGEV replication by shRNAs was dose-dependent.

3.3. Effective inhibition of TGEV RNA replication by shRNAs

To further confirm that the inhibition of TGEV-induced CPE resulted from homologous viral RNA degradation, a real-time

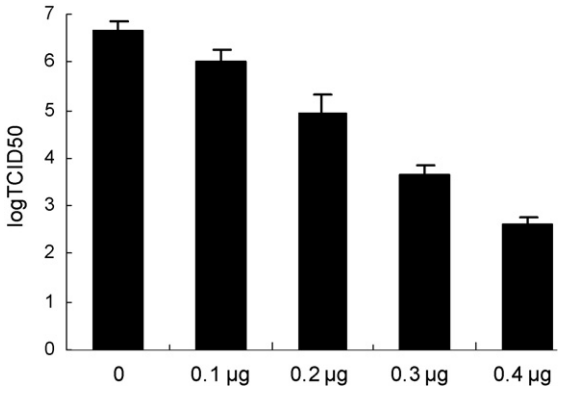

Fig. 3. Dose-dependent inhibition of TGEV replication. ST cells in each well were transfected with pEGFP-U6/P2 at the indicated doses, and then challenged with TGEV at 200 $CCID_{50}$. At 40 h post-infection, viral titers in these wells were measured and shown by log $CCID_{50}$/0.2 ml. Data shown are a mean of two independent experiments, and error bars represent the standard deviation.

quantitative RT-PCR analysis was performed. C_t values in three independent experiments were calculated by C_t method and the average of $2^{-\Delta\Delta C_t}$ value of viral RNAs in each sample was represented in Fig. 4. The data demonstrated that the relative amounts of viral RNAs in cell cultures transfected with pEGFP-U6/P1, pEGFP-U6/P2 and pEGFP-U6/T were 4.8, 0.01 and 84.5, respectively, where the amount of viral RNAs in mock control was regarded as 100. Analysis of these data revealed that the amounts of TGEV RNAs in samples transfected with pEGFP-U6/P1 and pEGFP-U6/P2 were reduced by approximately 95.2 and 100%, respectively. It suggested a potent inhibition of TGEV RNA replication triggered by sequence-specific shRNAs in ST cells.

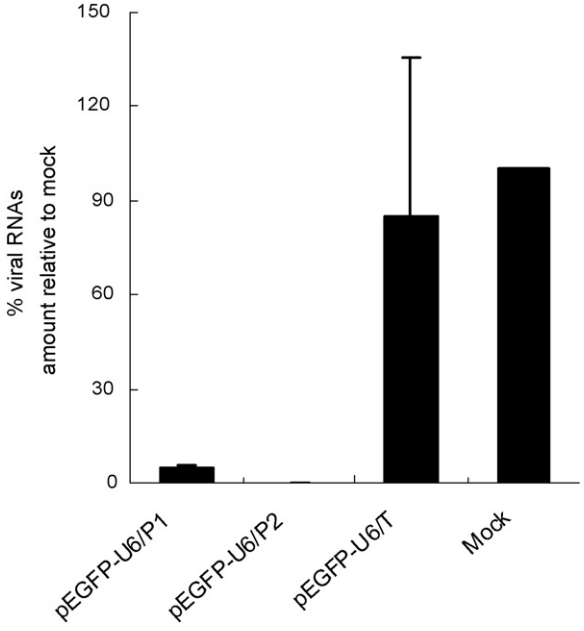

Fig. 4. Inhibition of TGEV RNA replication by shRNAs. ST cells were transfected with plasmids as indicated and then infected with TGEV. At 40 h after infection, cell cultures in the repeated wells were collected together for total RNAs extraction, and a real-time quantitative RT-PCR analysis was performed. The amount of viral RNAs in each sample was normalized to β-actin in the same sample, relative to the mock control. The mean of three independent transfections performed in triplicate is shown, and error bars represent the standard deviation.

4. Discussion

RNAi functions in a manner of sequence-specific, post-transcriptional gene silencing (PTGS) (Fire et al., 1998; Hannon, 2002). As the latest addition to the family of antisense technologies, it has emerged as a potentially important therapeutic strategy for antiviral activity in vitro and in vivo. Many of these kinds of studies have been focused on viruses infecting humans (Huelsmann et al., 2006; Li et al., 2005; Shlomai and Shaul, 2004; van Rij and Andino, 2006; Wu et al., 2005). Recently, however, several kinds of viruses infecting swine such as foot-and-mouth disease virus (FMDV), porcine circovirus (PCV) and porcine arterivirus (de los Santos et al., 2005; Liu et al., 2006; Lu et al., 2006) have also been silenced effectively. As TGEV is a porcine coronavirus with an RNA genome, it should be, as we have found, well sensitive to RNAi, too (van Rij and Andino, 2006).

Viral RdRp plays an essential role in viral RNA replication. Many researchers have shown that siRNA/shRNA targeting the RdRp coding gene could efficiently inhibit viral replication; moreover, according to their reports, viral inhibition triggered by siRNA/shRNA targeting the RdRp gene seems more efficient compared to other genes within the same genome (Ge et al., 2003; Lu et al., 2004; Wang et al., 2004). Therefore, the RdRp gene of TGEV, which is highly conserved among different strains, was employed as an RNAi target in this study, and our results demonstrated that two shRNAs generated from it both blocked viral replication with very high efficiency.

Examination of TGEV-induced CPE indicated that ST cells are very sensitive to TGEV infection. During the replication of TGEV, cell morphology changed quickly; therefore CPE analysis could be conducted directly on a phase-contrast microscopy without being further processed. On the other hand, the cell morphology changed so rapidly that almost the whole cell monolayer was lysed 40 h post-infection. As a result, the amount of total RNAs in the cell cultures of mock control decreased rapidly from then on. So, the cell cultures for viral titration and RNA extraction were harvested 40 h post-infection.

The CPE examination and MTS assay revealed that two sequence-specific shRNAs could trigger potent inhibition of TGEV replication. In fact, according to microscopic observation on duration of inhibition, ST cells transfected with pEGFP-U6/P2 (0.4 μg/well) were able to resist to CPE until the experiment was called off 72 h post-infection (data not shown). In their reports, several groups have demonstrated that siRNA/shRNA could apparently reduce virus-induced CPE in cell cultures (He et al., 2006; Mallanna et al., 2006; Wang et al., 2004); moreover, as we have shown (He et al., 2006) the inhibition is dose-dependent. A RT-qPCR analysis further confirmed that the efficient inhibition of viral replication was due to viral RNA degradation. In fact, as the pair of primers, designed for amplifying a region within N gene, could amplify all the

same regions within the TGEV genome RNA and subgenomic mRNAs (except the shortest one) simultaneously, the reduction of viral RNAs suggested that not only viral genome RNA itself, but also all its transcripts, required for viral structural protein expression, were decreased. Similar results have been obtained in other studies (Ge et al., 2003; Liu et al., 2006; Lu et al., 2004, 2006; Wang et al., 2004).

Except for the potent inhibition shown by two sequence-specific shRNAs, RT-qPCR analysis also demonstrated that, compared to mock control, the amount of viral RNAs in negative control decreased a little, too, which suggests a non-specific effect on TGEV replication in ST cells. Similarly, some groups have discussed an "off-target" effect induced by siRNA/shRNA in their reports. Scacheri et al. (2004) and Dahlgren et al. (2006) found no evidence showing that a double-stranded RNA-triggered IFN-associated antiviral pathway was activated or accounted for the non-specific effect, and Scacheri et al. (2004) further speculated that partial complementary sequence matching to "off-target" genes might result in a micro-RNA-like inhibition of translation. More recently, Jackson et al. (2006) confirmed his speculation by claiming that the "off-target" effect triggered by siRNA/shRNA was a ubiquitous phenomenon and resulted from short stretches of sequence complementary to the siRNA/shRNA seed region. Perhaps, according to these findings, there are some short stretches (<7 nt) complementary to TGEV RNA genome within the non-specific sequence (T) accounting for the non-specific inhibition; therefore, employing both a non-specific sequence and a heterologous virus as the negative control in a RNAi experiment may be a good idea [The porcine epidemic diarrhea virus (PEDV) CV777 strain was ever employed as a negative control in our study, and the poor inhibition of PEDV replication triggered by pEGFP-U6/P1 and pEGFP-U6/P2 indicated that shRNAs expressed from the two plasmids were TGEV specific (data not shown).]. Based on dose-dependent analyses, Liu et al. (2006) and Lu et al. (2006) found that, similar to sequence-specific inhibition, the non-specific effect also had a positive correlation with the concentration of shRNAs. In addition, during both dose-dependent and CPE analysis, we found that cell toxicity was dose-dependent, too. As the images shown in Fig. 1, when the amount of transfected plasmids reached 0.4 μg/well, cell morphology began to change and little more dead cells emerged in cultures in contrast to non-treated cells. As Couzin (2006) reported, the cell toxicity increased with the dose of plasmids increasing. Taken together, there may also be a correlation between cell toxicity and inhibition effect (both sequence-specific and non-specific) (Couzin, 2006; Fedorov et al., 2006). Anyway, compared to the slight non-specific effect, two sequence-specific shRNAs exhibited potent ability in silencing TGEV RNAs.

In conclusion, our results indicated that shRNAs targeting the RdRp gene in TGEV genome were capable of interfering with TGEV replication in ST cells with very high specificity and efficiency. This finding provides evidence that shRNAs could be used as a potential tool against TGEV infection in vitro. Whether the technology could be employed in vivo, in anti-TGEV therapy is still under investigation.

Acknowledgment

We would like to thank Mr. Yongqing Qian, from Shanghai Academy of Agricultural Sciences (China), for providing us the TGEs-1 strain and his generous help to this study.

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