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Short communication

Inhibition of porcine circovirus type 1 and type 2 production in PK-15 cells by small interfering RNAs targeting the Rep gene

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Abstract

Porcine circovirus type 1 (PCV1) and type 2 (PCV2) are two genotypes of porcine circovirus. Both of them are presumed to be widespread in the swine population. Currently, there is no specific treatment for their infections. RNA interference (RNAi) is a sequence-specific RNA degradation mechanism mediated by small interfering RNA (siRNA), which represents a possible therapeutic application for the treatment of viral infections. In this study, three siRNA expression plasmids (pS-RepA, pS-RepB and pS-RepC) were generated to target three different coding regions of the Rep protein (Rep) of PCV. These siRNAs were used to inhibit PCV production in a porcine kidney cell line, PK-15 cells. Our results revealed that Rep gene expression was inhibited by pS-RepA, pS-RepB and pS-RepC to different degrees. Moreover, our study also showed that the production of PCV1 and PCV2 was reduced by these siRNAs. pS-RepC, which targets the middle region of Rep gene, proved to be the most efficient siRNA for inhibition of Rep expression and viral production. Taken together, our data suggest that RNAi could be investigated as a potential treatment for PCV infection.

Keywords: Porcine circovirus; Small interfering RNA; Production

1. Introduction

Porcine circovirus (PCV), a member of the Circoviridae family, is a small, non-enveloped, spherical single-stranded DNA virus. There are two types of PCVs, namely PCV1 and PCV2. Both of them are presumed to be widespread in the swine population. Though PCV1 is considered to be nonpathogenic (Allan et al., 1995), it can infect human leukocytes in vitro, and is now considered to be a potential risk for viral transmission when porcine tissues and organs are used for xenotransplantation (Arteaga-Troncoso et al., 2005). PCV2 is associated

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with postweaning multisystemic wasting syndrome (PMWS) (Clark, 1997; Harding and Clark, 1997), porcine dermatitis, nephropathy syndrome (PDNS) (Rosell et al., 2000). Furthermore, PCV2 may damage the immune system and induce immunosuppression, which results in other pathogenyes coinfection (Segales et al., 2004). Although, vaccine for PCV2 has been developed recently, the efficiency still needs to be tested in the field trials. Thus, new method development for controlling PCV2 infection is urgently necessary.

The RNAs of PCV synthesized during productive infection in porcine kidney cells were recently characterized. During PCV1 replication, 12 species of RNAs are synthesized. These include capsid protein RNA (CR), eight Rep-associated RNAs (Rep, Rep′, Rep3a, Rep3b, Rep3c-1, Rep3c-2, Rep3c-3, Rep3c-4), and three NS-associated RNAs (NS462, NS642 and NS0). For PCV2, five Rep-associated RNAs (Rep, Rep′, Rep3a, Rep3b and Rep3c), and three NS-associated RNAs (NS515, NS672 and NS0), are synthesized during replication in PK-15 cells (Cheung, 2003a). Two proteins derived from ORF1, the full-length (Rep) and the spliced (Rep′), are essential for PCV DNA replication (Mankertz and Hillenbrand, 2001). It has been reported that mutations in Rep or truncated Rep′ proteins of PCV2 can cause more than a 99% reduction in viral protein synthesis and completely shut down viral DNA replication (Cheung, 2003b). It is, therefore, suggested that Rep or Rep′ could be a target for inhibition of PCV replication.

RNA interference (RNAi) was first discovered by plant biologists in the late 1980s. However, its molecular mechanism remained unclear until the 1990s, when an elegant work in the nematode *Caenorhabditis elegans* showed that RNAi is an evolutionarily conserved gene-silencing mechanism (Fire et al., 1998). The post-translational silencing of gene expression occurs in response to the introduction of homologous double-stranded RNA (dsRNA) into a cell. This phenomenon can result in highly specific suppression of gene expression. In the past few years, RNAi has become widely used as an experimental tool to analyze the function of genes and it represents a new feasible approach to develop effective antiviral treatments. RNAi antiviral therapy has been shown to inhibit the replication of human immunodeficiency virus (Capodici et al., 2002), severe acute respiratory syndrome virus (Wang et al., 2004), influenza virus (Ge et al., 2004) and foot-and-mouth disease virus (Chen et al., 2004). Therefore, RNAi would accord-

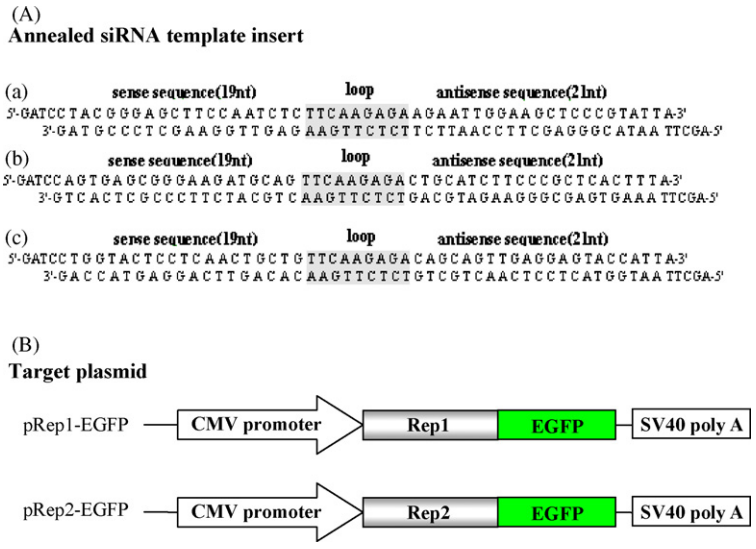

Fig. 1. Schematic representations of annealed siRNA template insert and target plasmid: (A) three pairs of two complementary 55-mer siRNA template oligonucleotides (a–c) were synthesized, annealed and inserted into the pSilencer4.1-CMV neo vector. (B) Rep1 and Rep2 gene were cloned into pCD-EGFP to fuse Rep1/Rep2 to the N terminus of enhanced green fluorescent protein (EGFP).

ingly serve as a new strategy for battling viral infections.

In this study, three siRNAs, specifically targeting the Rep gene RNA of both PCV1 and PCV2, were designed and used to inhibit the production of both viruses. Our results suggest that RNAi technology might serve as a potential molecular strategy for PCV therapy.

2. Materials and methods

2.1. Target sequence selection and vector construction

Three same target sequences (nts 130–150, 500–520 and 819–839 of PCV1; nts 143–163, 832–852 and 513–533 of PCV2) in the coding region of the Rep gene were selected and served as a basis for the design of the two complementary 55-mer siRNA template oligonucleotides (Fig. 1A). They were synthesized, annealed and inserted into the *Bam*HI and *Hin*dIII sites of the siRNA expression vector pSilencer4.1-cytomegalovirus (CMV) neo (Ambion). The resulting recombinant plasmids were designated as pS-RepA, pS-RepB and pS-RepC. The pSilencer4.1-CMV neo Negative Control (Ambion) is a negative control plasmid (pS-Negative) encoding a hairpin siRNA whose sequence is not found in the mouse, human or PCV genome databases.

The Rep gene of PCV1 (Rep1) and the Rep gene of PCV2 (Rep2) were amplified and subcloned into the vector pCD-EGFP, respectively, and then fused to the N terminus of the enhanced green fluorescent protein (EGFP) under the control of the immediate early promoter of human cytomegalovirus. The eukaryotic expression vectors were named pRep1-EGFP and pRep2-EGFP (Fig. 1B).

2.2. Cell culture and transfection

PCV-free PK-15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Shijiqing, China) at 37 °C and 5% CO_2. PK-15 cells were plated onto 6-well plates and cultured overnight. When cells reached 70–80% confluency, they were transfected separately with 6 μg of pS-RepA, pS-RepB, pS-RepC and pS-Negative in combination with 6 μg of pRep1-EGFP/pRep2-EGFP using LipofectamineTM 2000 (invitrogen) according to the manufacturer's recommendation. Non-transfected cells were used as negative controls.

2.3. Analysis of Rep and EGFP expression in PK-15 cells

Forty-eight hours after transfection, EGFP expression in the transfected cells was examined with the Olympus fluorescence microscope (Olympus, Japan). EGFP positive cells and EGFP expression signal were then evaluated by flow cytometry using the FACSCalibur Flow Cytometry System (BD, USA).

2.4. Virus titration by indirect immunofluorescence assay (IFA)

PCV2 (YU A strain) originally isolated from a lymph node tissue sample from a pig with naturally occurring PMWS (Cao et al., 2005) and PCV1 isolated from the IBRS-2 cell line (Cao et al., 2006) were used in this study. The $TCID_{50}$ of PCV was determined by IFA as previously described (Fenaux et al., 2002).

2.5. Viral challenge assay in PK-15 cells

Twenty-four hours after transfection with siRNA expression plasmids, the PK-15 cells were challenged with 500 $TCID_{50}$ of PCV1/PCV2 per 0.1 ml. After 1 h of adsorption, the inoculum was removed and cells were washed twice with DMEM. The infection was then performed in DMEM supplemented with 10% FBS. Seventy two hours after viral challenge, virus titer was detected by IFA. On the other hand, Rep mRNA level in the cells 48 h post-virus challenge and protein level of the capsid protein (Cap) in the cells 72 h post-virus challenge were evaluated by real-time RT-PCR and Western blotting, respectively.

2.6. Statistical analysis

Standard deviation of the mean and Student's *t*-test were determined using Microsoft Excel for all

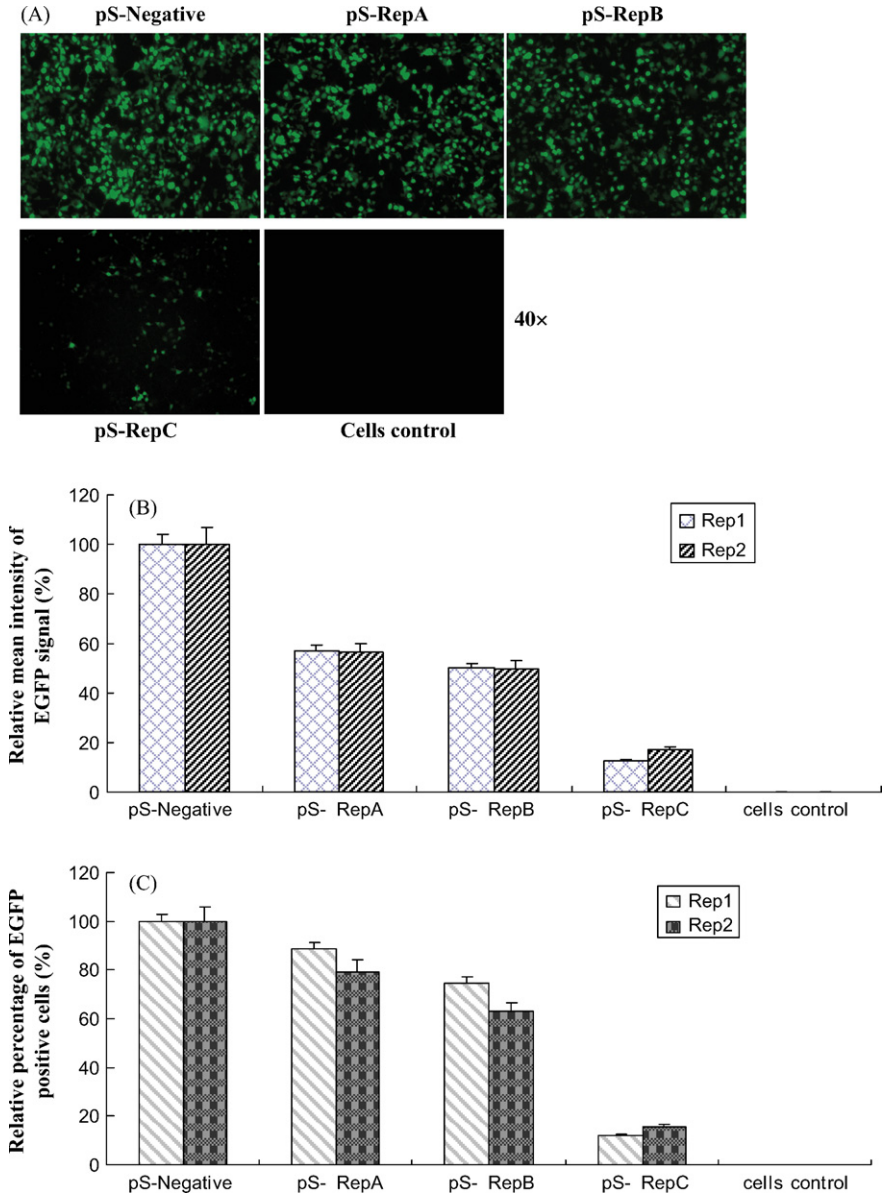

Fig. 2. Transient expression of siRNAs confers sequence-specific inhibition of expression of Rep1 and Rep2 in PK-15 cells: (A) EGFP expression in cells was evaluated with fluorescence microscopy 48 h post-transfection by pRep2-EGFP and pS-RepA, pS-RepB or pS-RepC, (B) the relative mean intensity and (C) relative percentage of EGFP positive cells in the cells cotransfected with pRep1-EGFP/pRep2-EGFP and siRNAs expression plasmids.

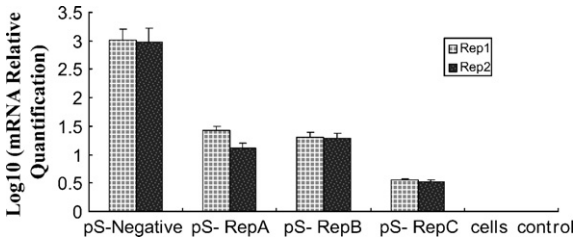

Fig. 3. Relative mRNA level of Rep in siRNA plasmid transfected cells was analyzed by real-time RT-PCR. The result showed that the Rep mRNA level was decreased quite significantly in the cells transfected with pS-RepC; ($P < 0.01$).

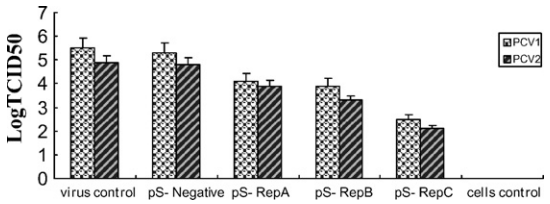

Fig. 5. The $TCID_{50}$ of PCV1 and PCV2 in the cells was evaluated by IFA. The results showed that both PCV1 and PCV2 production were inhibited significantly by pS-RepC; $P < 0.01$.

experiments, and each experiment was repeated at least three times.

3. Results

3.1. siRNA expression plasmids specifically inhibit Rep expression in PK-15 cells

We first examined the EGFP expression in the cotransfected cells using the Olympus fluorescence microscope (Olympus, Japan). Compared to the negative controls (cells cotransfected with pRep1-EGFP/pRep2-EGFP and pS-Negative), the green fluorescence signals were reduced in the cells cotransfected with pRep1-EGFP/pRep2-EGFP and pS-RepA, pS-RepB and pS-RepC, respectively. No EGFP signal was detected in the control cells (Fig. 2A). Furthermore, the inhibitory effects of the siRNAs on the expression of EGFP were quantitatively validated by flow cytometry analysis 48 h after transfection. The extent of EGFP down regulation was quantitated by assessing the mean fluorescence of the positive cells and the percentage of EGFP positive cells. As shown in Fig. 2B and C, for Rep1, the mean intensity of EGFP signal in the cells transfected with pS-RepC decreased about 87.9% ($P < 0.01$) (Fig. 2B), and the percentage of EGFP positive cells was reduced about 87.4% compared to that of negative controls ($P < 0.01$) (Fig. 2C). For Rep2, the mean intensity of the EGFP signal in the cells transfected with pS-RepC decreased about 84.5% ($P < 0.01$) (Fig. 2B), and the percentage of EGFP positive cells was reduced about 83.1% compare to that of the negative controls ($P < 0.01$) (Fig. 2C). For both Rep1 and Rep2, pS-RepA and pS-RepB resulted in a weak reduction in the mean intensity of EGFP signal (11.3 and 25.2% for Rep1; 20.7 and 37.1% for Rep2, $P < 0.05$) and the percentage of EGFP positive cells (43.1 and 49.8% for Rep1; 43.7 and 50.4% for Rep2, $P < 0.05$).

3.2. siRNA expression plasmids inhibit PCV viral production and Cap protein expression in PK-15 cells

To study the effect of siRNAs on viral transcription, mRNA levels of Rep1 and Rep2 in infected cells were

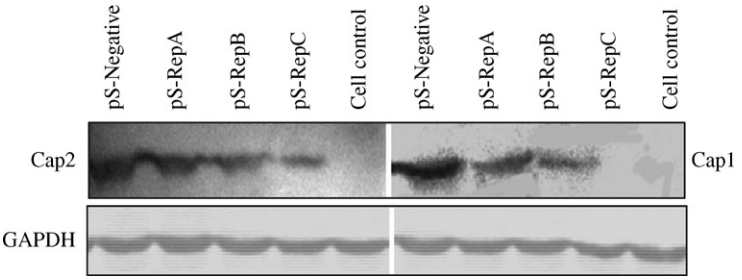

Fig. 4. Western blotting results showed that Cap protein expression of PCV was also inhibited to different degrees by siRNA plasmid transfection and pS-RepC was the most efficient siRNA plasmid for inhibition Cap protein expression.

detected by real-time RT-PCR (Fig. 3). The results showed that both Rep1 and Rep2 mRNA levels were reduced significantly in the cells transfected with pS-RepC ($P < 0.01$), a slight decrease was found in the cells transfected with pS-RepA and pS-RepB ($P < 0.05$). Interestingly, the expression of the Cap protein of PCV was also inhibited in the transfected samples. (Fig. 4). pS-RepC was the most efficient siRNA plasmid for inhibition of Cap expression suggesting that the inhibitory effects occur at both the transcriptional and post-transcriptional levels.

Seventy-two hours post-virus infection, virus titers in siRNA transfected cells and control cells were evaluated by IFA. In PCV1 infection, the mean virus $TCID_{50}$ in the cells transfected with pS-Negative, pS-RepA, pS-RepB and pS-RepC were $10^{5.3}$, $10^{4.1}$, $10^{3.9}$ and $10^{2.5}$, respectively, and in the cells without transfection, the $TCID_{50}$ was $10^{5.5}$ (Fig. 5). In PCV2 infection, the mean virus $TCID_{50}$ in the cells transfected with pS-Negative, pS-RepA, pS-RepB and pS-RepC were $10^{4.8}$, $10^{3.9}$, $10^{3.3}$ and $10^{2.1}$, respectively, and in the cells without transfection, the $TCID_{50}$ was $10^{4.9}$ (Fig. 5). The viral titers of cell lysates transfected with pS-RepC were reduced by approximately 1000-fold compared to that of the control group ($P < 0.01$). However, for both PCV1 and PCV2, the viral titers of cells transfected with pS-RepA and pS-RepB were reduced 10- and 100-fold ($P < 0.05$), respectively.

4. Discussion

PCV2 had been related to PMWS and other diseases, and PCV1 has been considered to be a potential risk for viral transmission when porcine tissues and organs are used for xenotransplantation (Arteaga-Troncoso et al., 2005). It is necessary to find an efficient method for PCV treatment. In this study, we used the eukaryotic expression vector pRep1-EGFP/pRep2-EGFP as a reporting system to monitor the function of the siRNAs. Rep1 and Rep2 expression were evaluated by fluorescence microscopy and flow cytometry. Our results revealed that pS-RepA, pS-RepB and pS-RepC specifically inhibited Rep expression and PCV production in PK-15 cells. Furthermore, we found that siRNAs which inhibited Rep expression can also decrease Cap protein expression in PCV infected cells. These results were in agreement with a recent study that found that shRNA could not only significantly reduce its corresponding mRNA level but inhibited other viral gene transcription as well (J. Liu et al., 2006). It is, therefore, assumed that siRNAs initiate the degradation of Rep mRNA, which then results in blockage of PCV replication and protein synthesis.

According to our data, pS-RepC, which targeting the middle site of the Rep gene RNA appeared much more efficient than pS-RepA and pS-RepB, which target the 5′ end and 3′ end of the Rep gene RNA, respectively. This indicates that the best target site seems to be close to the midpoint of the gene. Recently, siRNAs targeted against PCV2 infection in cells and mouse models were reported. The authors demonstrated that the siRNAs targeting the Rep gene of PCV2 at nts 624–642 was most effective (J. Liu et al., 2006). In our previous study, we also found that two siRNAs targeting the middle sequence of NS1 more efficiently inhibited Japanese encephalitis virus replication in BHK-21 cells (X. Liu et al., 2006). This phenomenon may be due to different positional accessibility caused by steric hindrance by a secondary or tertiary structure and/or protein binding (Yuan et al., 2005). In addition, analysis of ORF1 of PCV2 has shown 83% nucleotide and 86% predicted protein homology with PCV1. It suggests that siRNAs targeting same sequence of Rep1 and Rep2 may be efficient for both PCV1 and PCV2. In this study, we firstly reported that three siRNAs targeting the same sequence of Rep1 and Rep2 can inhibit Rep expression and viral production of PCV1 and PCV2.

Taken together, our research demonstrated that pS-RepA, pS-RepB and pS-RepC did not only effectively inhibit PCV Rep transcription and expression, but also inhibited PCV production in PK-15 cells. pS-RepC was most efficient for both PCV1 and PCV2.

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