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Adenovirus-mediated shRNA interference against porcine circovirus type 2 replication both in vitro and in vivo

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Abstract

Porcine circovirus type 2 (PCV2) is the primary causative agent of an emerging swine disease, postweaning multisystemic wasting syndrome (PMWS), which is responsible for the heavy economic losses in stockbreeding. There are no specific antiviral drugs for treatment of the virus infection. We have now constructed two recombinant adenoviruses expressing short-hairpin RNAs (shRNAs) directed against either ORF1 (rAdS1) or ORF2 (rAdS2) of PCV2 and measured the inhibition of PCV2 replication. The results showed that delivery of these shRNAs by recombinant adenovirus into PK15 cells could induce a significant inhibition of viral RNA and DNA replication and protein synthesis level in cells subsequently infected with PCV2. The antiviral effect was dose-dependent and could sustain at least for 120 h and the inhibition of virus replication could be significantly strengthened by combination of rAdS1 with rAdS2. Mice injected with shRNA before PCV2 infection showed substantial and low level of PCV2 DNA replication in the spleen during the period of 21–28 days post-PCV2 infection. These results indicated that shRNAs generated by adenovirus could sufficiently and continuously inhibit PCV2 infection in vitro as well as in vivo. The adenovirus based shRNA targeting ORF1 and ORF2 of PCV2 might be a new potential alternative strategy for controlling PCV2 infection.

Keywords: PCV2; RNAi; shRNA; Recombinant adenovirus

1. Introduction

Porcine circovirus type 2 (PCV2) has been recognized as the etiological agent of postweaning multisystemic wasting syndrome (PMWS) and reproductive failure in pigs (Clark, 1997; Gresham et al., 2000; Meehan et al., 2001), circulating in virtually all farms, with or without such syndrome (Larochelle et al., 2003). PCV2 is different from non-pathogenic porcine circovirus type 1 (PCV1), which was initially described as a contaminant of porcine cells (Tischer et al., 1982). Porcine diseases caused by PCV2, especially PMWS, have a significant impact on the economy of the pig industry worldwide. Although PCV2 vaccines have been developed (Pogranichnyy et al., 2004; Charreyre et al., 2005), the protective efficiency still needs to be tested in the field trials. Thus, it is imperative to develop new antiviral strategies with higher efficacy against the virus infection.

PCV2 is classified in the genus Circovirus of the Circoviridae family (Pringle, 1999). It has an approximately 1.76 kb single-stranded circular DNA genome and contains two major open reading frames (ORFs): ORF1 and ORF2. ORF2 encodes a viral caspid protein (Cap) involved in the host immune response (Mankertz et al., 1998; Nawagitgul et al., 2000). ORF1 encodes two replication-associated proteins (Rep and Rep′). Both Rep and Rep′ are absolutely essential for PCV2 replication. Mutations in Rep or truncated Rep′ proteins of PCV2 could cause >90% reduction in viral protein synthesis and completely shut down viral DNA replication (Cheung, 2003b).

RNA interference (RNAi) is a naturally occurring posttranscriptional gene silencing mechanism, which is induced by 19–27 nucleotide (nt) small interfering RNA (siRNA) molecules homologous to some region of the target gene (Jana et al., 2004). It has been found that this mechanism occurs in many organisms, such as plants, insects, epiphytes, metazoans and mammals (Ding et al., 2004; Gitlin and Andino, 2003). Because of the high rapidity and specificity of the RNAi effect, especially that mediated by a viral vector, it represents a new feasible approach to develop effective antiviral treatments. In

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recent years, RNAi technique has been used to induce gene silencing in a number of viruses, including foot-and-mouth disease virus (Chen et al., 2004), hepatitis B virus (HBV) (Zhou et al., 2005), influenza virus (Li et al., 2006), human immunodeficiency virus type 1 (Coburn and Cullen, 2002), human cytomegalovirus (Dittmer and Bogner, 2006), lymphocytic choriomeningitis virus (Sánchez et al., 2005) and herpes simplex virus (Muylaert and Elias, 2007).

Currently, the RNAi mediated by plasmid-borne short hairpin RNAs (shRNAs) has been used to inhibit PCV2 replication in PK-15 cells or BALB/c mice (Liu et al., 2006; Sun et al., 2007). Here we used recombinant replication-defective human adenovirus type 5 (rAd5) to deliver short hairpin RNAs (shRNAs) targeting ORF1 and ORF2 regions of PCV2, and found that they could inhibit PCV2 replication in both PK-15 cells and in BALB/c mice model.

2. Materials and methods

2.1. Cell and virus

The permanent PK15 cell line was maintained in RPMI-1640 (Gibco) supplemented with 8% heat-inactivated fetal calf serum (FCS) at 37 °C in a humidified 5% CO_2 incubator. Human kidney cells (AD-293) were used to generate and grow rAd5 and determine virus titers. The PCV2 strain SH, which was isolated from a pig with PMWS, was propagated in PK15 cells as previously described (Wang et al., 2007).

2.2. Design of shRNA and construction of recombinant plasmids

The target sequences were selected from the ORF1 and ORF2 region of PCV2 SH strain genome (NCBI GenBank; accession number: AY686763) using Ambion's web site tool to select potential siRNA (http://www.ambion.com/techlib/misc/siRNA_finder and http://www.oligoengine.com/Home/mid_prodSirna.html#sirna_tool). Two inverted repeats of siRNA target sequences, S1 (targeting ORF1) and S2 (targeting ORF2), were designed and chemically synthesized based on the protocol of the shRNA-expressing plasmids pSUPER (Oligoengene). The sequences of the fragments were as following: S1 (5′-GATCCCCACCACATACTGGAAACCACTT CAAGAGAGTGGTTTCCAGTATGTGGTTTTTTGGAAA-3′, sense) and S2 (5′-GATCCCCACT ACTCCTCCCGCCATA-CTTCAAGAGAGTATGGCGGGAGGAGTAGTTTTTTGGA-AA-3′, sense). They were subcloned into pSUPER at the BglII/HindII sites under the control of the PolIII human H1 RNA promoter (P_{H1}) and a termination signal of five thymidines (Ts), and named as pSUPER-S1 and pSUPER-S2, respectively.

2.3. Production of recombinant adenoviruses expressing short hairpin RNA

As shown in Fig. 1A, to get recombinant adenoviruses (rAd5), the fragment of Not I–HindIII of pSUPER-S1, pSUPER-S2 and pSUPER-H1 (as a control, the inserted sequence H1 was not related to PCV2) were cloned into the Not I/HindIII sites of adenovirus shuttle vector pAdTrack-CMV (Stratagene, La Jolla, CA) (He et al., 1998), and the resultants were designated as pAdTrack-S1, pAdTrack-S2 and pAdTrack-H1, respectively, in which the expression of shRNAs could be driven by either P_{H1} or by P_{CMV}. Then the vectors were linearized with Pme I and cotransformed by electroporation together with the adenoviral backbone plasmid pAdEasy-1 (Stratagene) into *E. coli* BJ5183. The recombinant adenoviral plasmids were generated by homologous recombination. Positive clones were selected and confirmed by making DNA minipreps and digesting with *Pac* I. The linearized adenoviral plasmids (pAdS1, pAdS2, and pAdH1) (Fig. 1B) were purified by ethanol precipitation, and transfected into AD-293 packaging cells that had been plated in a 25-cm^2 flask the previous day. The cells were transfected with 5 μg of linearized plasmid DNA using 20 μl of Lipofectamine reagent 2000 (Invitrogen) and monitored for expression of green fluorescent protein (GFP). To generate high-titer viral stock, adenoviruses rAdS1, rAdS2, and rAdH1 (as a control) were harvested and passaged 3 times in AD-293 cells. Final virus yields were $10^{9.5}$ efu (expression-forming units)/ml.

2.4. Cell transfection and viral challenge

The inoculation of rAd5 was performed under optimal conditions. Briefly, PK15 cells, a cell line susceptible to rAd5 infection but not permitting productive replication, were seeded and incubated at 37 °C overnight. When cells reached 80–90% confluency, they were inoculated with rAd5 at multiplicity of infection (MOI) of 500 in RPMI-1640 medium with 2% fetal calf serum. At 24 h post-inoculation, PCV2 was added at 100 MOI without removing the rAd5 suspension. Twenty-four hours later, cells were additionally treated with 300 mM D-glucosamine as described previously (Tischer et al., 1987). At 48 h after PCV2 infection, cells were analyzed by IFA, and supernatants and cell lysates were collected for real-time PCR and Western blotting analysis, respectively.

2.5. Real-time PCR

Newly synthesized viral DNA in cell culture was assayed by real-time PCR as described elsewhere (Feng et al., 2006). Briefly, the supernatants of infected PK15 cells at 72 h post-inoculation with rAd5 were used as templates for real-time PCR amplification. The sense primer PCV2F: 5′-CCAGGAGGGCGTTCTGACT-3′, the antisense primer PCV2R: 5′-CGTTACCGCTGGAGAAGGAA-3′ and the probe 5′-FAM-AATGGCATCTTCAACACCCGCCTC-TARAM-3′ were used for amplification of PCV2 DNA. Quantitative real-time PCR was carried out using the ABI7300 v.1.3 (ABI).

2.6. Semi-quantitative RT-PCR

Total cell RNAs were prepared from virus-infected PK15 cells 72 h after being inoculated with rAd5 using Trizol RNA Extract reagent (Invitrogen). cDNAs were

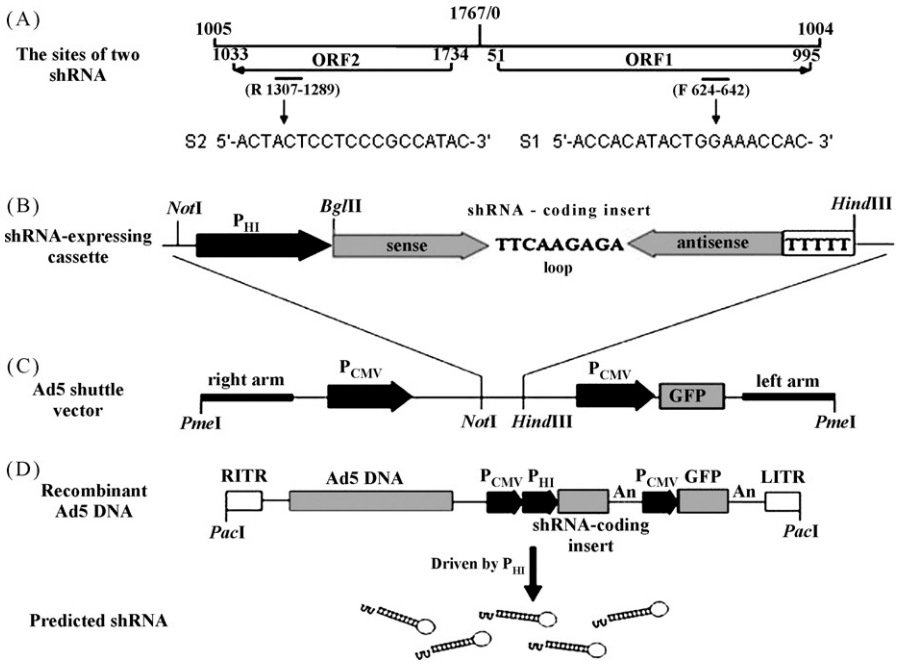

Fig. 1. Sites of two shRNA, shRNA-expressing cassette Ad5 shuttle vector and rAd5 DNA. (A) Schematic representation of the sites of the two shRNA target sequences against genomic DNA of PCV2 strain SH. (B) An inverted repeat corresponding to each of the target sequences in the PCV2 genome was inserted under the control of P_{H1} and a transcriptional termination signal of five Ts. (C) The shRNA-expressing cassette was then subcloned into the multiple cloning sites of Ad5 shuttle vector pAdTrack-CMV under the control of P_{CMV} and a poly(A) transcription termination signal (An). (D) The resultant Ad5 shuttle vector was cotransfected with the adenoviral backbone plasmid pAdEasy-1 into *E. coli* by electroporation. The recombinant adenoviral DNAs were generated by homologous recombination. As a result, transcription of the shRNA-coding insert could be driven by P_{H1}. The synthesized RNAs should therefore fold back to form shRNAs that are finally processed into the putative siRNAs.

reverse transcribed from 2 μg total RNAs using OligodT_{18} primer and MLV-RT (Promega). Semi-quantitative RT-PCR was performed with 2 μl cDNA using following primers: Forf1, 5′-ATGCCCAGCAAAAAGAATGG-3′, and Rorf1, 5′-CCCACAATGACGTGTTCATTG-3′, for PCV2-ORF1; Forf2, 5′-GGTTTGTAGCCTCAGCCAAAGC-3′ and Rorf2, 5′-GCACCTTCGGATATACTGTCAAGG-3′, for PCV2-ORF2; and Fcyclo, 5′-TAACCCCACCGTCTTCTT-3′, and Rcyclo, 5′-TGCCATCCAACCACTCAG-3′, for cyclophilin. The PCR consisted of an initial enzyme activation step at 95 °C for 5 min followed by 24 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR products were electrophoresed in 1.5% agarose gel and photographed.

2.7. Indirect fluorescence assay (IFA)

PK15 cells were seeded and infected with PCV2. After 48 h post-infection (hpi), cells were fixed in methanol and washed with phosphate-buffered saline (PBS). After fixation, the cells were blocked by PBS with 3% BSA at RT for 1 h. The porcine anti-PCV2 antibody, diluted in PBS containing 3% BSA (PBSB) (1:50), was added to the cells and incubated at 37 °C for 1 h. After washing with PBS containing 0.1% Tween-20, an optimum dilution (1:100) of fluorescein-conjugated goat anti-mouse antibody was added and incubated for 1 h at 37 °C. After washing, the cells were examined under a fluorescence microscope. Cells positive for PCV2 viral antigens were counted in six fields of view.

2.8. Western blotting

Western blot analysis was performed after the cells were harvested by centrifugation at 500 × *g* for 8 min. The pellets were lysed for 10 min on ice in 150 μl lysis buffer [20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate]. Lysates were then collected by centrifugation at 14,000 × *g* for 10 min at 4 °C and the concentration was measured with the Bradford method (Bradford, 1976). Twenty micrograms of total cellular protein from each sample was subjected to 12% SDS-PAGE and transferred to nitrocellulose membrane (Stratagene). The membranes were blocked for 4 h at RT in blocking buffer TBST (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.1% Tween-20) containing 10% skim milk powder to prevent nonspecific binding and then incubated with porcine anti-Cap antibody (obtained after vaccinating pigs with purified Cap protein expressed in *E. coli* system) and rabbit anti-β-actin antibody (Bios) at RT for 2 h. Then, the membranes were washed three times with TBST and incubated for 1 h at RT with peroxidase-Protein A (Boster Co., China) diluted in blocking buffer (1:10,000). After washing, the objective proteins on the membrane were revealed by an enhanced chemiluminescence with SuperSignal West Pico Trial Kit (Pierce Co., China).

2.9. Viral challenge assay in BALB/c mice

One hundred BALB/c mice of 56-day-old were randomly assigned into five groups of 20 animals each. Group 1 inoculated

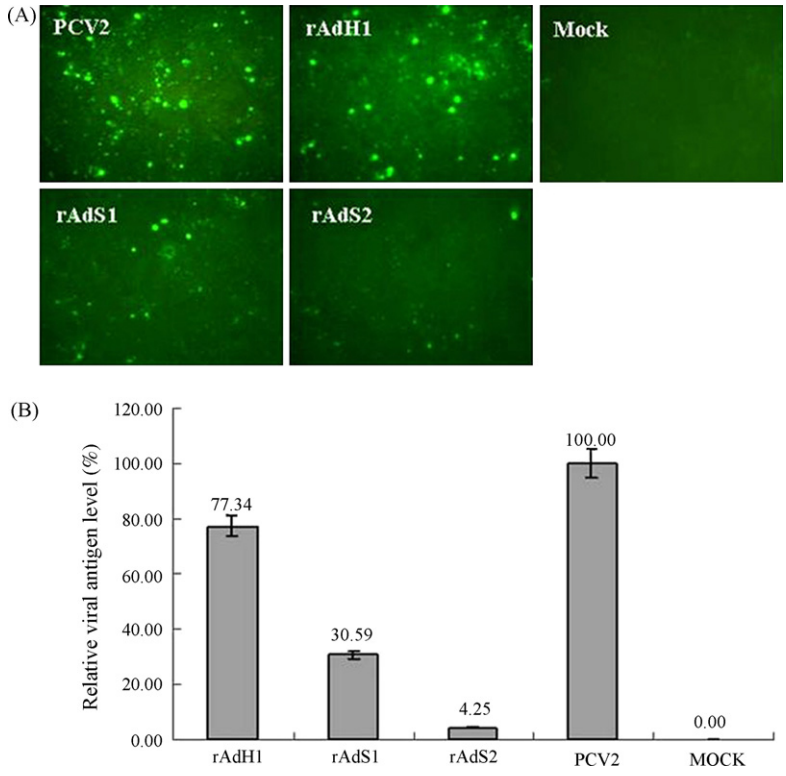

Fig. 2. Inhibition of PCV2 replication in cell culture by different shRNA-expressing rAd5s. (A) The PK-15 cells treated with rAd5s were infected with PCV2 at an MOI of 100. After 48 h incubation cells were immunostained by IFA with porcine anti-PCV2 antibody. (B) The amounts of PCV2 viral antigen are shown as percentages of the PCV2-positive signals in the PCV2 (alone)-infected group (positive control). Error bars represent deviation of the median from three experiments.

with PBS was served as mock. Groups 2–4 were inoculated via both nasal and celiac routes with recombinant adenovirus rAdS1, rAdS2 and rAdH1 ($10^{8.0}$ efu per mouse), respectively. Group 5 served as positive control (PCV2 infected alone). After 24 h, all mice in Groups 2–5 were each challenged intranasally and interperitoneally with $10^{4.0}$ $TCID_{50}$ of PCV2. The animals were monitored daily for clinical signs of disease until to 28 days post-challenge (DPC). Every week post-infection, five mice were randomly selected from each group and euthanized. Then the necropsy was performed and spleens were collected for PCV2 DNA examination in the tissues.

2.10. Statistical analysis

Statistical comparisons were made by using Student's *t* test, and the differences between groups were considered significant if the *P* value was <0.05.

3. Results

3.1. Inhibition of PCV2 replication in PK-15 cells by rAd5 expressing PCV2-specific shRNA

To test whether shRNA could inhibit PCV2 replication in cell culture, PK15 cells were infected with the shRNA-expressing recombinant virus and then challenged with PCV2. The numbers of PCV2-infected cells in PK15 cells were determined by IFA. As shown in Fig. 2, both rAdS1 and rAdS2 markedly reduced the frequency of fluorescence-stained cells compared with those in the rAdH1 control and PCV2 only control groups ($P < 0.05$). The rAdS2 exhibited significant higher level of inhibition than did rAdS1 ($P < 0.05$).

3.2. Inhibition of PCV2 replication is dose-dependent

A dose–response analysis was conducted by incubating PK15 cells with increasing amounts (1–1000 MOI) of rAdS1, rAdS2 or rAdH1 control. The numbers of PCV2 antigen cells were measured by IFA at 48 h after PCV2 infection. The results showed that the levels of PCV2 antigen cells were significantly decreased in a dose-dependent fashion in the presence of rAdS1and rAdS2 ($P < 0.05$). Inoculating rAdS2 at a MOI of 1000 resulted in the highest (approximately 25-fold) inhibition of viral replication compared with positive control (Fig. 3).

3.3. Inhibition of PCV2 viral transcription, DNA replication and protein synthesis in PK-15 cells by shRNAs

To confirm that the effect of rAdS1 and rAdS2 on PCV2 viral production was through the reduction of PCV2 RNA levels, we performed semi-quantitative RT-PCR analysis using RNA extracted from PK15 cells at 72 h after inoculation with rAd5. The amount of each viral mRNA was normalized to that of cyclophilin mRNA in the same sample. The abundance of ORF1 and ORF2 mRNAs was significantly reduced in PCV2-infected cells inoculated with rAdS1 or rAdS2 (Fig. 4A). The

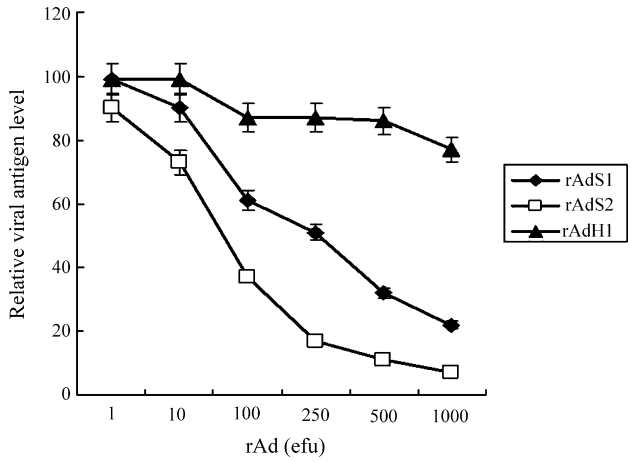

Fig. 3. Dose-dependent inhibition of PCV2 protein synthesis. PK15 cells were inoculated with the indicated amounts of rAd5s and followed by infection with PCV2 at an MOI of 10 and assayed for the amount of PCV2 viral antigen at 48 h post-infection by IFA. The amounts of PCV2 viral antigen are shown as percentages of the PCV2-positive signals in the PCV2 (alone)-infected group (positive control). Data shown were from one of the two experiments.

results from the RNA control samples were all negative (data not shown).

To further study the inhibitory effect of rAdS1 and rAdS2 on PCV2 DNA replication, the supernatants of infected PK15 cells were treated and assayed by real-time PCR. The results indicated that the amount of PCV2 DNA had a significant down-shift in the treated cells with rAdS1 (62.88%) or rAdS2 (90.91%) compared to that of the control PCV2-infected cells ($P < 0.05$) (Fig. 4B).

In addition, PCV2 Cap protein production was detected by Western blotting at 48 h after PCV2 infection. As shown in Fig. 4C, Cap protein synthesis was suppressed by both rAdS1 and rAdS2, and more efficiency was observed in rAdS2 treated group.

3.4. Cooperated inhibition of PCV2 replication by two shRNAs

To confirm whether the inhibition was stronger when PK15 cells were inoculated with both rAdS1 and rAdS2, PK-15 cells were infected with rAdS1 or/and rAdS2 at an MOI of 500 each. To keep the same amount of rAd5 virus were seeded in every group, another 500 MOI of rAdH1 were added into the single rAd5-infected groups. PCV2 production in every group was determined by IFA at 48 h after PCV2 infection. As shown in Fig. 5, in the group of combination of treatment with rAdS1 and rAdS2, the inhibition of virus replication was significantly increased as compared to rAdS1 and rAdS2 individually ($P < 0.05$).

3.5. shRNA sustains inhibition of PCV2 replication in PK-15 cells

The duration of inhibition of PCV2 caused by rAdS1 and rAdS2, were measured by IFA at 24, 48, 72, 96 and 120 h post-infection of PCV2. As shown in Fig. 6, in rAdH1control groups, it resulted in no significant reduction of virus yield, and viral production reached maximum level at 72 h pi. The rAdS1 and rAdS2 resulted in a marked reduction of virus yield by approximately 20 and 100-fold, respectively, compared with the control (PCV2 only) ($P < 0.05$). The effects extended to 120 h post-infection.

3.6. PCV2 suppression by shRNA in previously infected PK-15 cells

To test if shRNAs could suppress PCV2 in previously infected cells, cells were infected with PCV2 at a MOI of 100 and passaged for 3 times. Then the cells were seeded into cell-plates and incubated at 37 °C. When cells reached 80–90% confluency, they were inoculated with rAd5 at a MOI of 500. At 48h post-

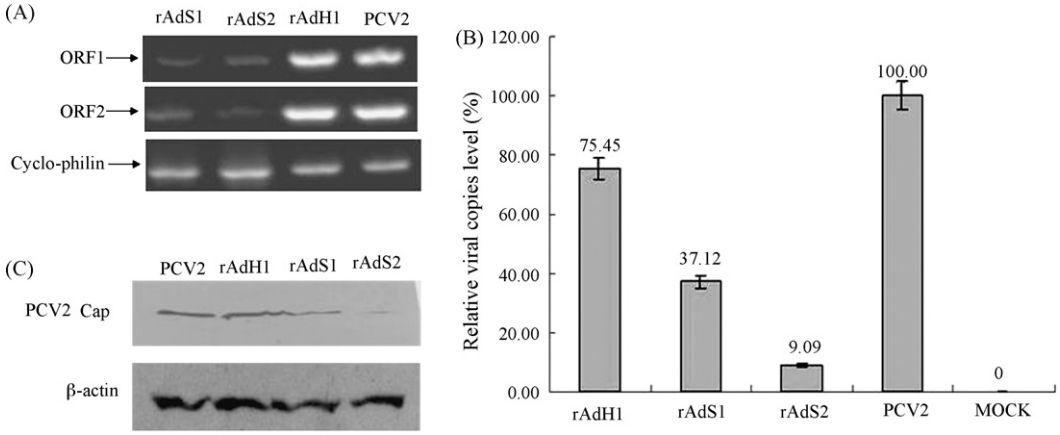

Fig. 4. Inhibition of PCV2 viral transcription (A), DNA replication (B) and protein synthesis (C) by shRNA-expressing rAd5s in cell culture. (A) Total RNAs isolated from PCV2-infected cells 72 h after inoculation with rAd5 were subjected to a semi-quantitative RT-PCR analysis of ORF1 and ORF2 mRNAs. The data were normalized based p, the amount of cyclophilin mRNA. (B) Total DNA extracted from the supernatants of PK15 cells treated with rAd5 followed by PCV2 infection 48 h post-infection of PCV2 were used as template for real-time PCR to analysis PCV2 DNA. The data were expressed as percentages of the normalized value for the PCV2 (alone)-infected cells (positive control). Data shown were from one of the two experiments. (C) Whole-cell extracts prepared from treated PK15 cells 72 h after inoculated with rAd5 were assayed by Western blotting for PCV2 protein expression in PCV2-infected cells. β-actin was used as the loading control.

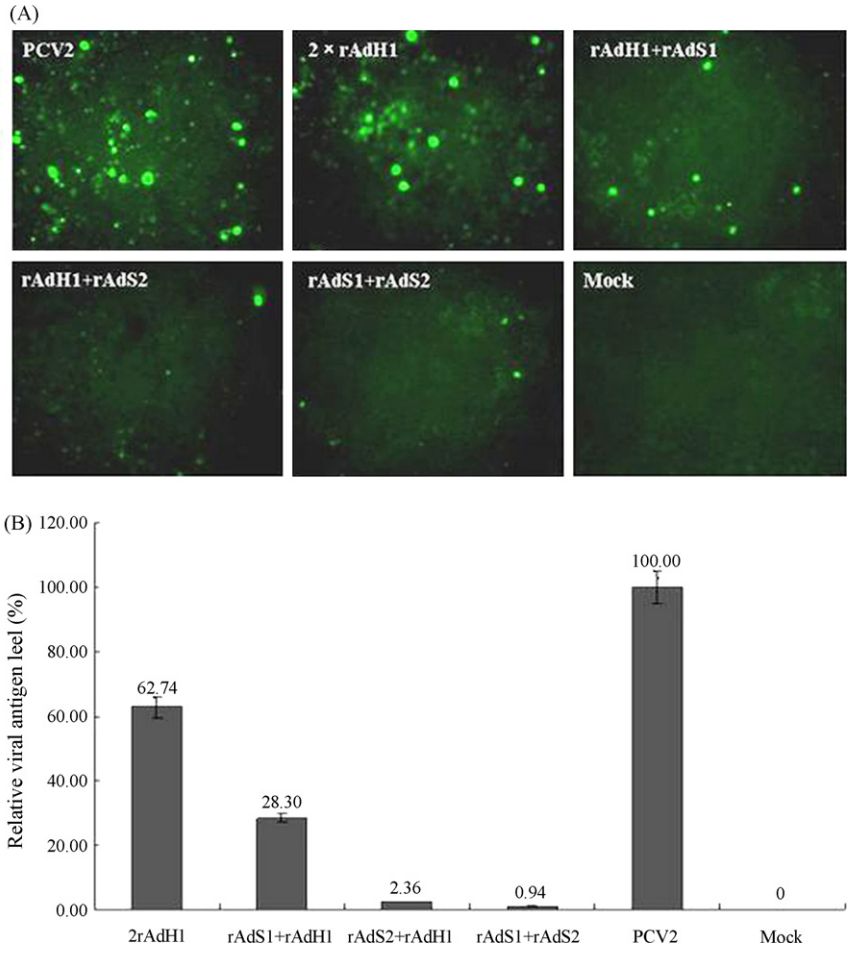

Fig. 5. Inhibition of virus replication by rAdS1 and rAdS2 alone and in combination. (A) PK-15 cells were inoculated with rAdS1 or/and rAdS2 at an MOI of 500 each and followed by PCV2 infection with an MOI of 100. At 48 h post-PCV2-infection, cells were immunostained by IFA with porcine anti-PCV2 antibody. (B) The amounts of PCV2 viral antigen are shown as percentages of the PCV2-positive signals in the PCV2 (alone)-infected group (positive control). Data shown are from one of two experiments.

incubation, the levels of PCV2 protein synthesis and DNA were measured by IFA and real-time PCR, respectively. As shown in Fig. 7, rAdS1 and rAdS2 in pre-infected cells were able to inhibit viral replication significantly ($P < 0.05$).

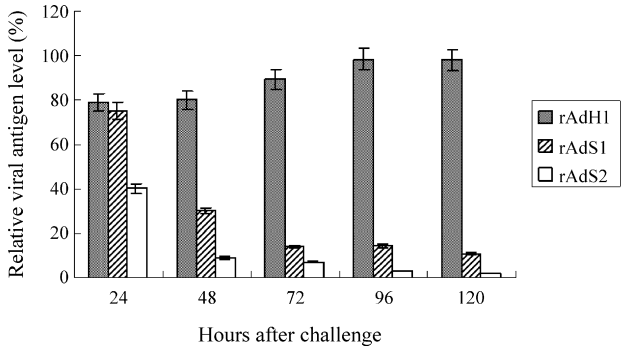

Fig. 6. Kinetics of inhibition of PCV2 protein synthesis by shRNAs. PK15 cells were inoculated with rAd5 at an MOI of 500. At 24 h, they were inoculated with PCV2 at an MOI of 100. The cultures were incubated for 12, 24, 48, 72, 96 or 120 h and at the end of the incubation time and the levels of PCV2 were measured by IFA. The amounts of PCV2 viral antigen are shown as percentages of the PCV2-positive signals in PCV2 (alone)-infected cells (positive control). Data shown are from one of three experiments.

3.7. Inhibition of PCV2 DNA replication in mice by shRNA

At 7, 14, 21, and 28 days post-infection, no clinical signs and gross lesions were observed in all groups. The results of real-time PCR showed that PCV2 DNA persistently increased in the spleen in the PCV2-alone-infected group and rAdH1 control group, and reached the highest level (4×10^5 copies/μg DNA) at 21 DPC. However, at 21 and 28 days post-infection, the virus yields in rAdS1-treated group were reduced by 91.4 and 94.6%, respectively, and the virus yields in rAdS2-treated group were decreased by 96.8 and 96%, respectively. It indicated that both rAdS1 and rAdS2 could significantly inhibit replication of PCV2 in mice at 21 and 28 days post-infection, as compared to the control (PCV2)-infected group ($P < 0.05$) (Fig. 8).

4. Discussion

RNAi has been developed into a powerful tool to down-regulate the expression of endogenous or exogenous nucleic acid sequences within the cell (Morris et al., 2004). Functional siRNA or shRNA, which cause gene silencing, can be synthesized chemically (Randall et al., 2003), or delivered by plasmid

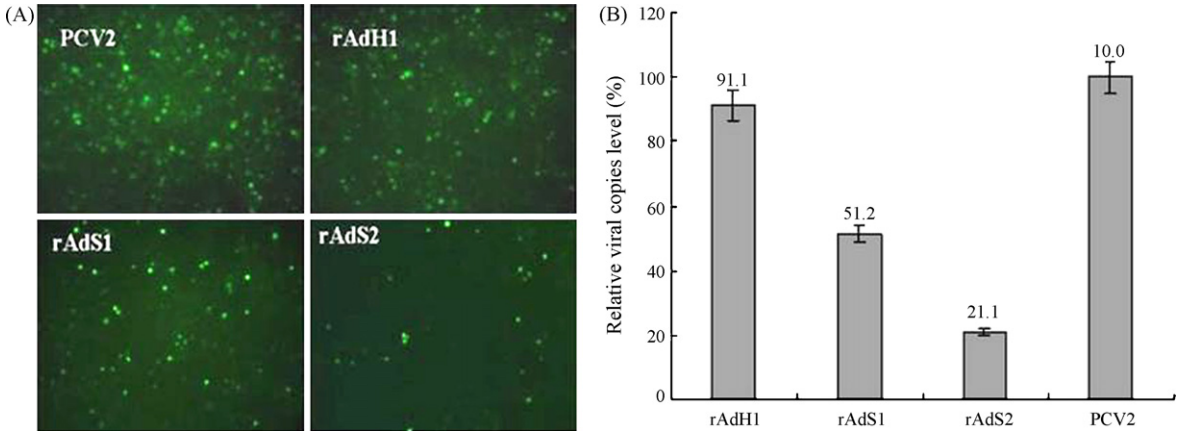

Fig. 7. Inhibition of PCV2 replication by shRNA in PK-15 cells pre-infected with PCV2 virus. Cells infected with PCV2 were passaged 3 times, and then were treated with an MOI of 500 of rAd5s. After 48h rAd5-treatment, cells were immunostained by IFA with porcine anti-PCV2 antibody (A). The supernatants of the cells cultures above were used as template for real-time PCR to analyze PCV2 DNA, and the data shown are the percentages of the normalized value for PCV2 (alone)-infected cells (positive control) (B). Data shown are from one of the three experiments.

vector (Sui et al., 2002) and viral vector expression cassettes (Moore et al., 2005). Viral vector expression cassettes easily can deliver shRNA into cells with high efficiency and induce steady interference with long duration; they can be potentially used as a therapeutic or prophylactic means against viruses (Haasnoot et al., 2003). Here, we firstly designed and constructed two recombinant replication-defective human adenoviruses expressing shRNAs targeting the ORF1 and ORF2 gene of PCV2, and found that they could induce highly efficient inhibition of PCV2 replication both in PK-15 cells and in mice.

Usually, RNAi operates at the post-transcription level to suppress gene expression in cell (McManus and Sharp, 2002). During PCV2 replication in PK15 cells, nine PCV2-specific RNAs are synthesized, including the capsid RNA, five Rep-associated RNAs and three NS-associated RNAs (Cheung, 2003a). Some researchers found that the RNAi generated by plasmid-borne short hairpin RNAs (shRNAs) could inhibit PCV2 replication in DNA, RNA and protein level (Liu et al., 2006; Sun et al., 2007). In the present study, we showed that the inhibition efficiency could be signifi-

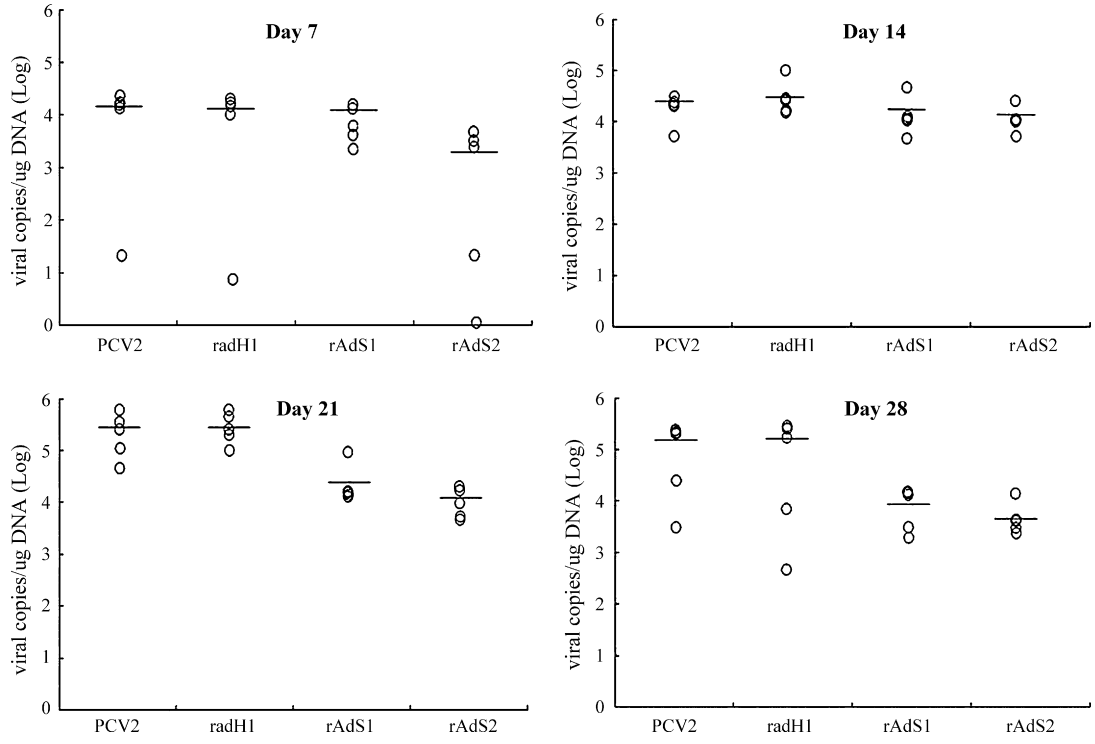

Fig. 8. Effects of shRNA on PCV2 viral replication in mice. Twenty BALB/c mice in each group were injected with rAd5 via both nasal and celiac routes at $10^{8.0}$ efu per mice. At 24 h post-injection, each mouse was infected with $10^{4.0}$ $TCID_{50}$ PCV2 via the same routes. At 7, 14, 21 and 28 days after PCV2 infection, the PCV2 DNA amount in spleen of mouse were detected by real-time PCR and showed as circle (◯) in the figures. Average DNA amount of five mice in each group at one time-point is shown as horizontal line (—).

cantly enhanced by the combination of rAdS1 with rAdS2. In addition, we noticed that rAdH1, as a control, has some inhibitory effect on PCV2 replication as shown in Figs. 2, 3, 4B and 5B. Possibly, the high concentrations of siRNA exhibited non-specific gene suppressive activity (Persengiev et al., 2004).

Previous studies have shown that some viruses are able to counteract RNA interference by producing proteins that suppress this process at different levels (Ding et al., 2004; Roth et al., 2004). To address whether this phenomenon existed in PCV2 replication in PK15 cells, we evaluated the capacity of the RNAi response to silence PCV2 replication when the rAd5s were inoculated into cells after infection with PCV2. The results showed that PCV2 replication was suppressed even when the shRNAs were added at 24 h post-infection with PCV2, a time point at which viral protein synthesis is already predominant in the infected cells. It suggested that the RNAi machinery was active and effective even in the presence of previously synthesized viral proteins, and thus those viruses did not encode an RNAi suppressor. However, additional experiments are clearly needed to explain this observation.

To test the ability of rAdS1 and rAdS2 to inhibit replication of PCV2 in vivo, BALB/c mice were used as a model in this study. The results indicated that the virus load could be significantly reduced in the spleen of mice treated by rAdS1 or rAdS2 during a period of 21–28 days compared with that in PCV2 control ($P < 0.05$). However, under the condition of real-time PCR, PCV2 could not be detected in the serum obtained from the animals at any point measured throughout the entire post-challenge period. In addition, no obvious microscopic lesions in lung or spleen were observed in mice challenged with PCV2 (data not shown), which was not consistent with the previous report by Kiupel et al. (2001). The possible reason might be related to the PCV2 strain, the breed of mice or the sensitivity of real-time PCR under the conditions used. Nevertheless, the results of the animal experiment confirmed that the two recombinant adenoviruses expressing the shRNA could suppress PCV2 replication efficiently in vivo.

In summary, here, we identified the shRNAs generated by recombinant adenoviruses (rAdS1 and rAdS2) targeting ORF1 and ORF2. The viral vector-based RNAi technology could specifically inhibit PCV2 replication in both PCK15 cells and mice. Inoculation of recombinant adenovirus expressing shRNA might be a potential new method in the prophylaxis and therapy of PCV2 infection.

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