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Inhibition of porcine reproductive and respiratory syndrome virus replication by adenovirus-mediated RNA interference both in porcine alveolar macrophages and swine

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) has been mainly responsible for the heavy economic losses in many swine-producing regions. Current vaccination strategies and antiviral drugs provide only limited protection. Consequently, there is a need to develop a new antiviral strategy. In this study, two recombinant adenoviruses expressing short-hairpin RNAs (shRNAs) directed against ORF1b of PRRSV S1 strain were constructed and the inhibition of PRRSV replication was determined. The results showed that pretreatment with these shRNAs delivered by recombinant adenovirus could induce a significant inhibition of viral RNA and protein level in Marc-145 cells infected with PRRSV S1 strains. One recombinant adenovirus (rAd-P2) was found to be also effective in inhibiting the replication of highly virulent PRRSV SY0608 strain in Marc-145 cells and porcine alveolar macrophages at both the protein and ORF1b mRNA level. The antiviral effect was dose-dependent and sustained for at least 96 h. Twenty 6-week old piglets were assigned to four groups each with five piglets. Groups 1 and 2 were inoculated intramuscularly with rAd-P2 and mock construct rAd-mP2 individually. After 24 h, groups 1, 2 and 3 were challenged intramuscularly with the SY0608 strain. Group 4 remained unchallenged but with PBS as mock. The results showed that the viral load of PRRSV in serum and lung tissue of swine was suppressed effectively by rAd-P2. The clinical signs and pathological lesions in the pigs inoculated with rAd-P2 were milder than those in rAd-mP2 negative and PRRSV control. These results indicated that shRNAs mediated by the adenovirus could inhibit PRRSV infection sufficiently *in vitro* as well as *in vivo*. RNAi mediated by recombinant adenovirus might be a potential new tool for controlling PRRSV infection. Of course, the protective efficiency of rAd-P2 should be made by using a large number of pigs in future.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically significant viral diseases in the swine industry, which characterized by reproductive problems in sows such as poor farrowing rates, premature farrowings, and increased stillbirths, as well as respiratory problems in piglets such as pneumonia and atrophic rhinitis (Collins et al., 1992; Wensvoort et al., 1991). Recently, a highly pathogenic PRRSV spread wildly in many swine herds in China and caused serious economic losses (Li et al., 2007b; Tian et al., 2007). The current commercial PRRSV vaccines do not sufficiently protect pigs from PRRSV infection. Therefore, it is imperative to develop new antiviral strategies to prevent and control this viral infection.

PRRSV is an enveloped, single-stranded positive-sense RNA virus of the family *Arteriviridae* in the order *Nidovirales*. The genome of PRRSV is approximately 15 kb in length and consists of nine open reading frames (ORFs) (Dea et al., 2000; Wu et al., 2001). Among them, ORF1a and ORF1b are situated at the 5′ end of the genome, represent nearly 75% of the viral genome, and encode proteins with apparent replicase and polymerase activity. The ORF7, situated at the 3′ end of the genome, encodes a 14–15 kDa capsid protein (N) constituting about 20–40% of the proteins of the virion (Mounir et al., 1995; Nelson et al., 1995). PRRSV can persist in pigs for a long period of time after initial infection and the infected animals can shed infectious virus for several months, even though the pigs have PRRSV-specific humoral and cell-mediated immune responses (Christopher-Hennings et al., 1995; Mateu and Diaz, 2008).

RNA interference (RNAi) is a natural posttranscriptional gene silencing mechanism, which is induced by 19–27 nucleotide (nt) small interfering RNA (siRNA) molecules homologous to the target genes (Jana et al., 2004). Many studies have shown that short-

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hairpin RNA (shRNA) could suppress gene expression significantly when delivered into mammalian cells *in vitro* (Dykxhoorn et al., 2003; McManus and Sharp, 2002). In recent years, the RNAi technique has been used to induce gene silencing in a number of viruses, including foot-and-mouth disease virus (Chen et al., 2006), hepatitis B virus (Uprichard et al., 2005), influenza virus (Tompkins et al., 2004), porcine circovirus type 2 (Feng et al., 2008) and human immunodeficiency virus (An et al., 2007). It has been demonstrated that shRNAs directed against ORF1b, ORF5 and ORF7 regions of PRRSV could markedly inhibit virus replication in Marc-145 cells by using plasmids expressing shRNA (Huang et al., 2006; Li et al., 2007a). However, the potential of shRNA to prevent PRRSV infection or suppress PRRSV replication *in vivo* has not been established. In this study, two recombinant adenoviruses expressing shRNA targeting ORF1b of PRRSV were constructed and it was found that they could inhibit PRRSV replication efficiently in Marc-145 cells, porcine alveolar macrophages and piglets.

2. Materials and methods

2.1. Cell cultures and viruses

Human embryo kidney cells (HEK-293A) were used to generate recombinant adenovirus type 5 (rAd5) and to determine virus titers. Marc-145 cells were used to grow PRRSV and to determine virus titers. HEK-293A cells and Marc-145 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of heat-inactivated fetal bovine serum (FBS). Cells were trypsinized and seeded in 96-well or 24-well plates at 20- to 24 h before virus inoculation. Porcine alveolar macrophages were obtained from 3 to 5-week old piglets (serologically negative of PRRSV and PCV2) and were cultured in RPMI-1640 supplemented with 10% FBS as described previously (Zeman et al., 1993). Macrophages were seeded at the rate of 10^6 cell/ml in 96- or 24-well plates and were allowed to adhere for 7 h. Non-adherent cells were decanted, and wells were refilled with RPMI medium and 10% FBS. Inoculation was done at 24 h post-seeding. All the cultures were incubated at 37 °C with 5% CO_2. The high virulent PRRSV strain SY0608 (15 passages) [GenBank accession number EV144079] and virulent strain S1 (30 passages) [GenBank accession number DQ459471] were isolated and stored in the laboratory, and were used for viral challenge.

2.2. Construction of recombinant plasmids and production of recombinant adenovirus

The shRNA-expressing plasmids pSUPER-P2, pSUPER-P3 and pSUPER-mP2 were constructed as described previously (Huang et al., 2006; Li et al., 2007a). pSUPER-mP2 which had three different nucleotides from the wild-type shRNA, pSUPER-P2, was used as a negative control (Fig. 1A). To construct recombinant adenovirus shuttle vectors, the primers containing *Kpn* I enzyme site in the sense primer and *Xho* I in the antisense primer were used. P_{H1}-shRNA-expression cassettes were amplified from the pSUPER constructs, respectively, and then inserted into the *kpn* I/*xho* I sites of pAdTrack-CMV (Stratagene, La Jolla, CA) (He et al., 1998). The resultant recombinant shuttle vectors, in which the expression of shRNAs could be driven by both P_{H1} and P_{CMV}, were confirmed by sequencing and designated as pCMV-P2, pCMV-P3 and pCMV-mP2, respectively (Fig. 1).

The recombinant shuttle vectors were linearized with Pme I and cotransformed with the pAdEasy-1 into *E. coli* BJ5183 by electroporation. Positive clones were selected and confirmed by making DNA minipreps and Pac I digestion. The resulting adenoviral plasmids were linearized with Pac I, purified by ethanol precipitation, and transfected into HEK-293A cells. The cells in each well were transfected with 1 μg of linearized plasmid DNA using 3 μl of Trans Fast™ Transfection Reagent (Promega). The recombinant adenovirus yields of rAd-P2, rAd-P3 and rAd-mP2 (negative control), were confirmed by the expression of green fluorescent protein (GFP). To generate high-titer viral stock, adenoviruses rAd-P2, rAd-P3, and rAd-mP2 were harvested and passaged three times in HEK-293A. Final virus yields were $10^{9.0}$ $CCID_{50}$/ml.

2.3. Viral challenge in vitro

To assess the capacity of rAd5 to inhibit PRRSV replication *in vitro*, Marc-145 cells and porcine alveolar macrophages (PAMs), susceptible to rAd5 infection but not permitting productive replication,

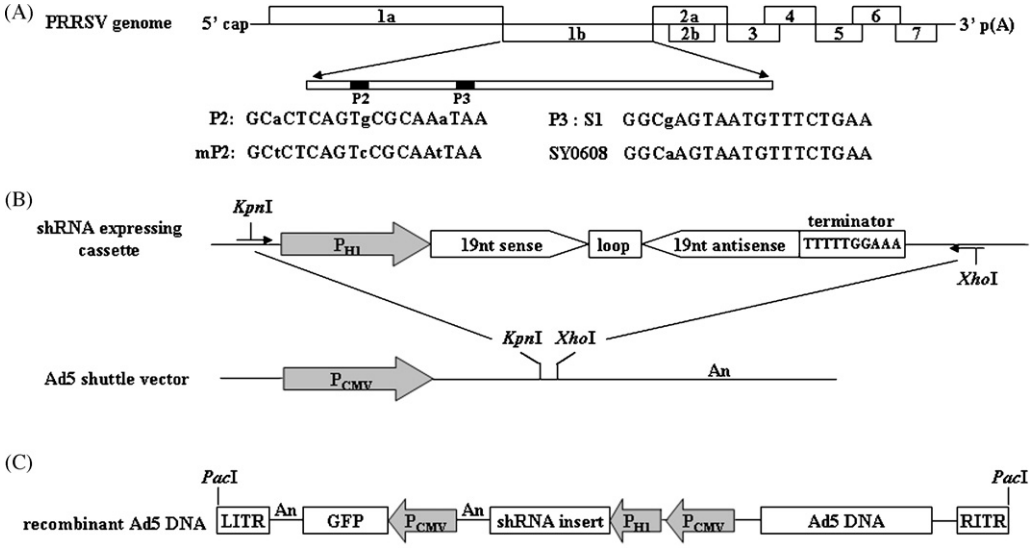

Fig. 1. Schematic description of target viral genome, shRNA-expressing cassette, Ad5 shuttle vector and rAd5 DNA. (A) Genomic structure of PRRSV and position of target siRNA: P2 and P3. S1 and SY0608 contain a nucleotide difference within the P3 targeted sequence. mP2 had three different nucleotides from P2 was used as negative control. Mismatches are indicated by lowercase letters. (B) An inverted repeat directed to the target site in PRRSV genome was cloned into vector under the control of P_{H1} and a transcriptional terminator. The shRNA-expressing cassette was amplified using PCR and subcloned into Ad5 shuttle vector pAdTrack-CMV under the control of P_{CMV} and a poly(A) transcriptional termination signal (An). The transcription of shRNA could be driven by either P_{CMV} or P_{H1}. (C) The resultant Ad5 shuttle vector was cotransfected with the adenoviral backbone plasmid pAdEasy-1 into BJ5183 by electroporation, and the recombinant adenoviral DNAs were generated by homologous recombination.

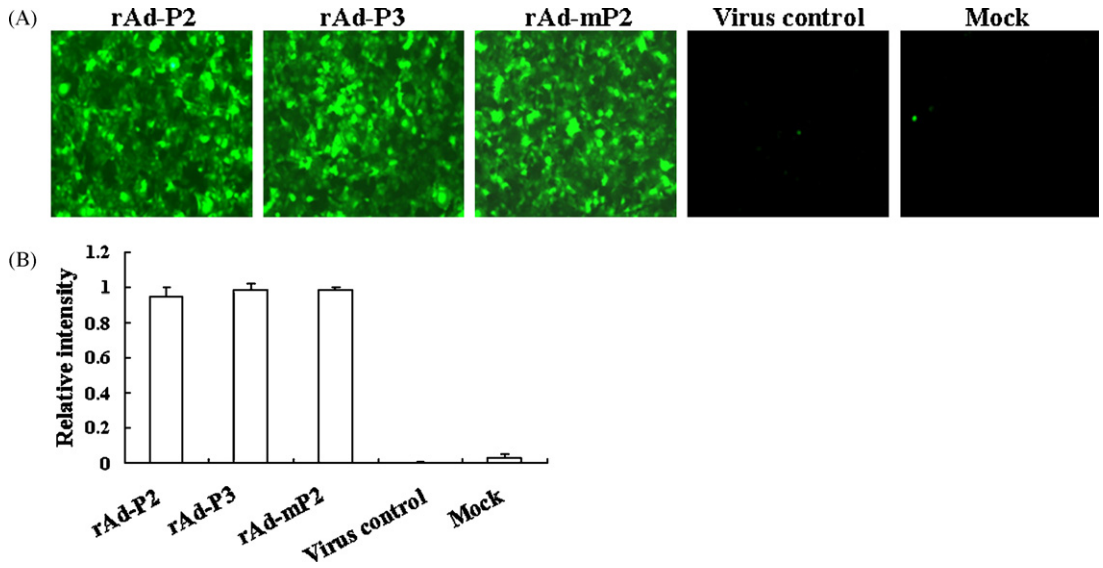

Fig. 2. Quantification of GFP intensity in Marc-145 cells. The fluorescence in each well was first observed under an inverted fluorescence microscope (A), and the relative level of GFP intensity was measured by chemiluminescence's detector (B). The pictures were taken with an inverted fluorescence microscope. Magnification, 200×. The data shown here was one of three experiments.

were seeded and incubated overnight at 37 °C with 5% CO_2. When the cells reached 80–90% confluency, they were inoculated with rAd5 at a multiplicity of infection (MOI) of 500 in RPMI-1640 medium with 2% fetal calf serum. At 24 h post-inoculation, the cells were challenged with PRRSV at a MOI of 10 without removing the rAd5 suspension. At 48–72 h post-challenge (hpc), the cells were examined microscopically for GFP expression and cytopathogenic effects (CPE). The relative level of GFP expression in the cells was measured by a chemiluminescence's detector using Gene5 software (BioTek Instruments, Inc., Winooski, VT). Briefly, the OD_{485} values in the wells inoculated with different rAd5 were examined individually. After averaging the score from images of each treatment, the score for background (non-rAd5 treatment) was subtracted to generate the data presented in Fig. 2. The cells were analyzed by IFA, and the supernatants and cell lysates were collected for real-time PCR and Western blotting analysis, respectively.

2.4. Indirect immunofluorescence assay (IFA)

Marc-145 cells or macrophages seeded in 96-well plates were fixed in ethanol. Following three washes in phosphate-buffered saline (PBS, pH 7.4), the fixed cells were incubated with monoclonal antibody (McAb) against the N protein (kindly provided by Dr. D.A. Benfield and E.A. Nelson in South Dakota University, Vermillion, SD) and mice serum antibody against ORF1b of PRRSV SY0608 (made with the recombinant protein expressed in *E. coli* in the laboratory) for 1 h at 37 °C. Unbound antibody was washed three times with PBS containing 0.1% Tween-20. Fluorescein-conjugated goat anti-mouse antibody (Boster Bio-Tech Co. Ltd., Wuhan, China) was added and incubated for 1 h at 37 °C. After three washes with PBS, positive signal was analyzed under fluorescence microscopy (Zeiss Axiovert 200).

2.5. Analysis of PRRSV ORF1b gene in Marc-145 cells and macrophages by real-time PCR

Total RNA was extracted from Marc-145 or macrophages cultures with TRIzol® Reagent (Invitrogen, Carlsbad, CA). Reverse transcription was carried out using MLV-RT (Promega, Madison, WI) in 25 μl reaction mixture containing 2 μg RNA according to the Manufacturer's instructions. One microliter of RT reaction mixture was submitted to real-time PCR analysis by using ORF1b-specific primers (sense: 5′-CCT AAT GCT ACT CCG GTG ACT GA-3′; antisense: 5′-TGG TCG GCA CAT ACA ACT CAA-3′), and SYBR® Green Realtime PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan), according to the Manufacturer's recommendations. The reaction procedure was 95 °C 2 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. β-actin gene served as an internal reference (Li et al., 2007a). Cycle times of the internal reference that varied by >1.0 unit in triplicate were discarded. The relative amount of target gene mRNA was normalized to that of β-actin mRNA in the same sample. To confirm specific amplification, melting curve analysis of the RT-PCR products was performed according to the Manufacturer's protocol. Real-time PCR was performed in an ABI PRISM 7300 sequence detection system and analyzed with ABI PRISM 7300_{SDS} software (Applied Biosystems, Foster City, CA).

2.6. Quantitative RT-PCR for PRRSV in serum and lung tissues

Total RNA was extracted from serum and lung tissues as above and ORF7 mRNA of PRRSV SY0608 was detected as described previously (Huang et al., 2006). For each assay a standard curve was generated using serially diluted PRRSV SY0608 standard of 10^0–10^8 $CCID_{50}$/ml.

2.7. Western blotting assay

Marc-145 cells plated in six-well plates were harvested in lysis buffer (50 mM Tris–HCl, pH 8, 6.25 mM EDTA, 1% NP-40, 0.4% deoxycholate) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by blotting onto nitrocellulose (NC) membrane (Pall Co., Ann Arbor, MI). The membrane was incubated with anti-N monoclonal antibody and rabbit anti-β-actin antibody (Bios Biotech Co., Ltd, Beijing, China), followed by incubation with peroxidase-conjugated goat anti-mouse antibody or peroxidase-conjugated SPA. The proteins were visualized by enhanced chemiluminescence (Denville Scientific Inc., Metuchen, NJ).

2.8. Viral challenge assay in pigs

Twenty 6-week old piglets (Duroc × (Landrace × Domestic breeds), weighing 12.5–15.0 kg) free from PRRSV, PRV and PCV-2

were randomly divided into four groups of five animals each and housed separately in disease-secure isolated facilities. Group 1 and 2 were inoculated intramuscularly with recombinant adenovirus rAd-P2 and rAd-mP2 (4×10^9 $CCID_{50}$ per piglet) individually, while the group 3, untreated with rAd5, was used as PRRSV challenge control. Group 4 was inoculated with PBS and served as mock. After 24 h, all the pigs in groups 1–3 were challenged intramuscularly with $2 \times 10^{4.0}$ $CCID_{50}$ of PRRSV SY0608 (15 passages). The animals were monitored daily for body temperature and clinical signs of disease until 9 days post-challenge (dpc). On 1 and 5 dpc, the blood and serum samples were collected for PRRSV titration using quantitative RT-PCR. On 9 dpc, all piglets were euthanized and necropsied, and the lung tissues were collected for viral RNA and pathological examinations.

2.9. Pathological examinations

All the piglets were necropsied and the macroscopical lung lesions of the animals were evaluated in a blind manner based on the approximately volume that each lobe contributes to the entire lung as described by Halbur et al. (1995). The sections (5 μm thick) of 10% neutral buffered formalin-fixed, paraffin-embedded lung tissues were processed for hematoxylin and eosin (HE) staining. Interstitial pneumonia was evaluated based on the increased thickness of the alveolar walls, accumulation of mononuclear inflammatory cells and proportion of infected tissue.

2.10. Statistical analysis

Results were analyzed for significance by one-way ANOVA test using SPSS software version 16.0. The mean difference was considered significant at the 0.05 level.

3. Results

3.1. rAd5 expressing shRNA inhibit replication of different PRRSV isolates in Marc-145

Two recombinant adenoviruses expressing shRNA directed against different regions of ORF1b gene of PRRSV S1 were constructed and designated as rAd-P2 and rAd-P3, respectively. It was found that there was a single mismatched nucleotide between shRNA expressed by rAd-P3 and the target site in ORF1b gene of new highly virulent strain SY0608, based on sequence analysis (Fig. 1). Another recombinant adenovirus rAd-mP2 which had three different nucleotides from the wild-type shRNA expressed by rAd-P2 was constructed and used as a negative control. All three rAd5 recombinants could express GFP in cells at 48 h post-infection (Fig. 2A).

In order to investigate whether the replication of the high pathogenic strain SY0608 and the traditional strain S1 could both be inhibited by rAd-P2 and rAd-P3, Marc-145 cells were challenged with PRRSV 24 h post-inoculation with the rAd5 individually. After incubation for 48 h, the cells were examined for GFP expression and CPE, and the culture supernatants were collected and the virus yield was determined by $CCID_{50}$. The results showed that the frequency of GFP expressing cells was almost the same in the wells inoculated with the rAd5 individually (Fig. 2). In the presence of rAd-P2 and rAd-P3, there was no CPE induced by PRRSV S1, and the titer of PRRSV S1 was reduced significantly by approximately 100–1000-fold, respectively ($P < 0.05$). When challenged with SY0608, only rAd-P2 could prevent CPE from SY0608 infection effectively, and the titer of PRRSV SY0608 was reduced significantly by approximately 100- to 1000-fold ($P < 0.05$) (Fig. 3). Thus, rAd-P2 was competent to suppress the replication of the two PRRSV strains, and it was selected in the following studies in which only SY0608 strain was used for challenge.

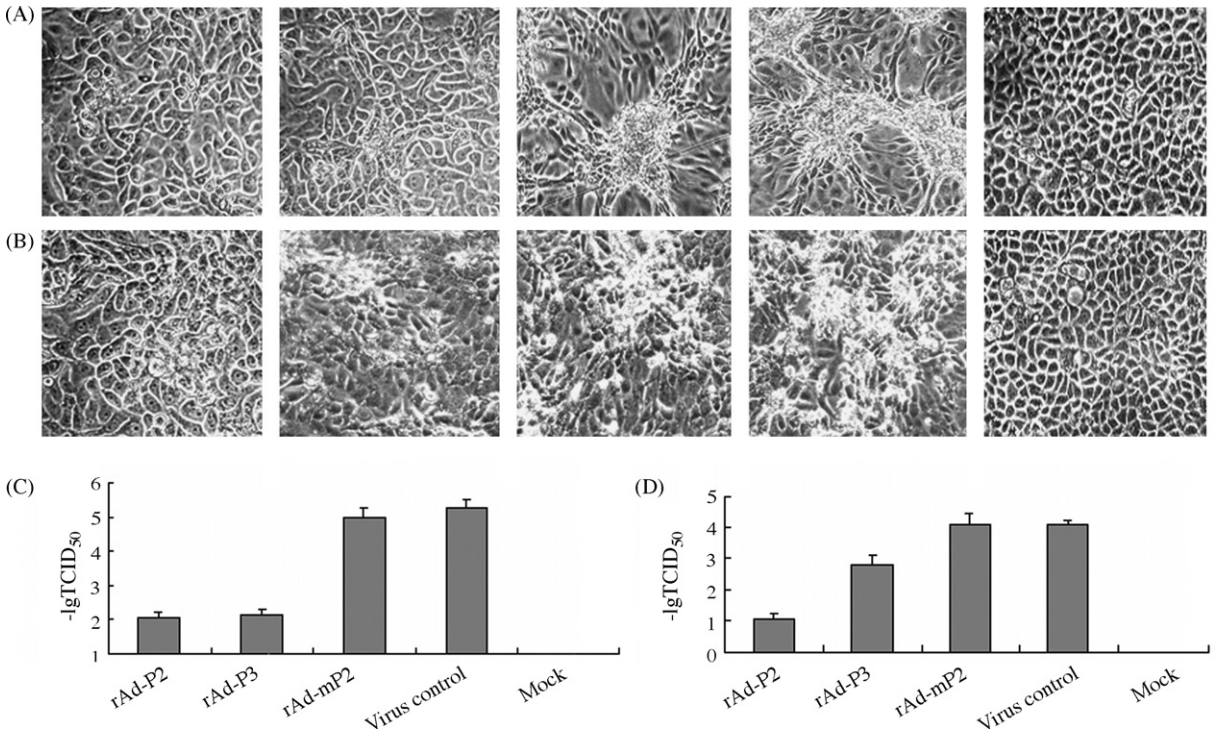

Fig. 3. Inhibition of PRRSV S1 (A and C) and SY0608 (B and D) replication by rAd5 in Marc-145 cells. Cells were challenged with PRRSV (at 10 MOI) at 24 h after inoculation with rAd-P2, rAd-P3 or rAd-mP2 at 500 MOI. After 48–72 h incubation, the cells were examined for CPE (A and B) and then culture supernatants were collected and virus yields were detected by $CCID_{50}$ (C and D). The pictures were taken with an inverted fluorescence microscope. Magnification, 200×. The data shown was one of three experiments.

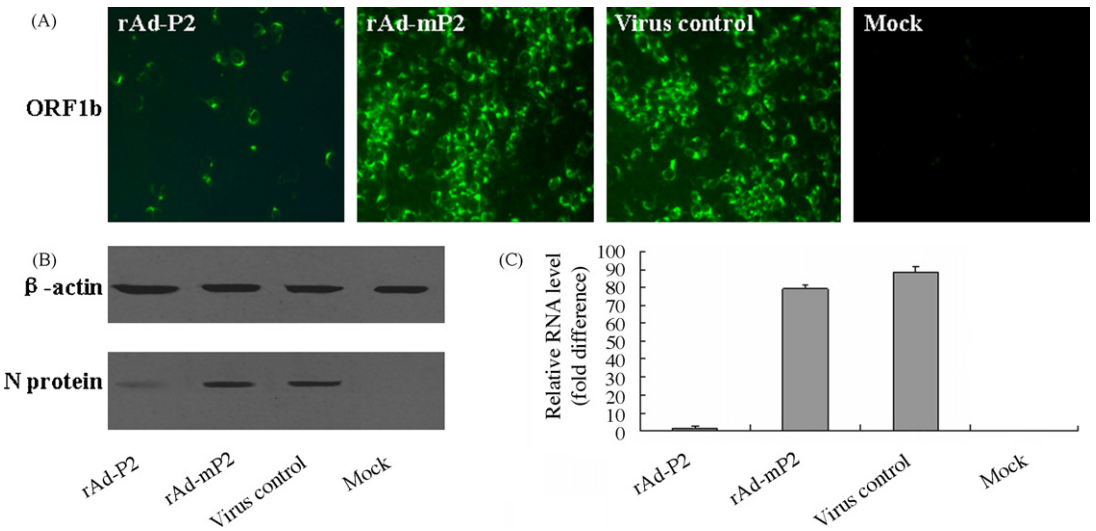

Fig. 4. Suppression of PRRSV SY0608 replication by rAd-P2 in Marc-145 cells at different levels. Cells seeded in 96-well plates were inoculated with rAd5 at 500 MOI, and challenged with PRRSV SY0608 (at 10 MOI) at 24 h post-inoculation. At 72 hpc, cells were fixed and PRRSV were detected by IFA with antibody to ORF1b (Magnification, 200×) (A). In other experiments, cells were collected and lysed for Western blotting analysis with anti-N monoclonal antibody and anti-β-actin monoclonal antibody (B), and cell cultures were collected for determination of the ORF1b mRNA level by quantitative RT-PCR. The mRNA of β-actin served as an internal reference and the level of ORF1b mRNA was normalized to the level of β-actin mRNA in the same sample (C). The data shown here was from one of three experiments.

3.2. rAd-P2 suppresses replication of SY0608 strain in Marc-145 at different levels

Cells treated with rAd-P2 were challenged with PRRSV and detected by IFA, Western blotting analysis using monoclonal antibody against N protein or antibody against ORF1b protein, and ORF1b mRNA level was determined by real-time PCR. As shown in Fig. 4, ORF1b and nucleocapsid (N) protein level of PRRSV were depressed by rAd-P2 as detected by IFA and Western blot. ORF1b mRNA level of PRRSV was decreased significantly by rAd-P2 in

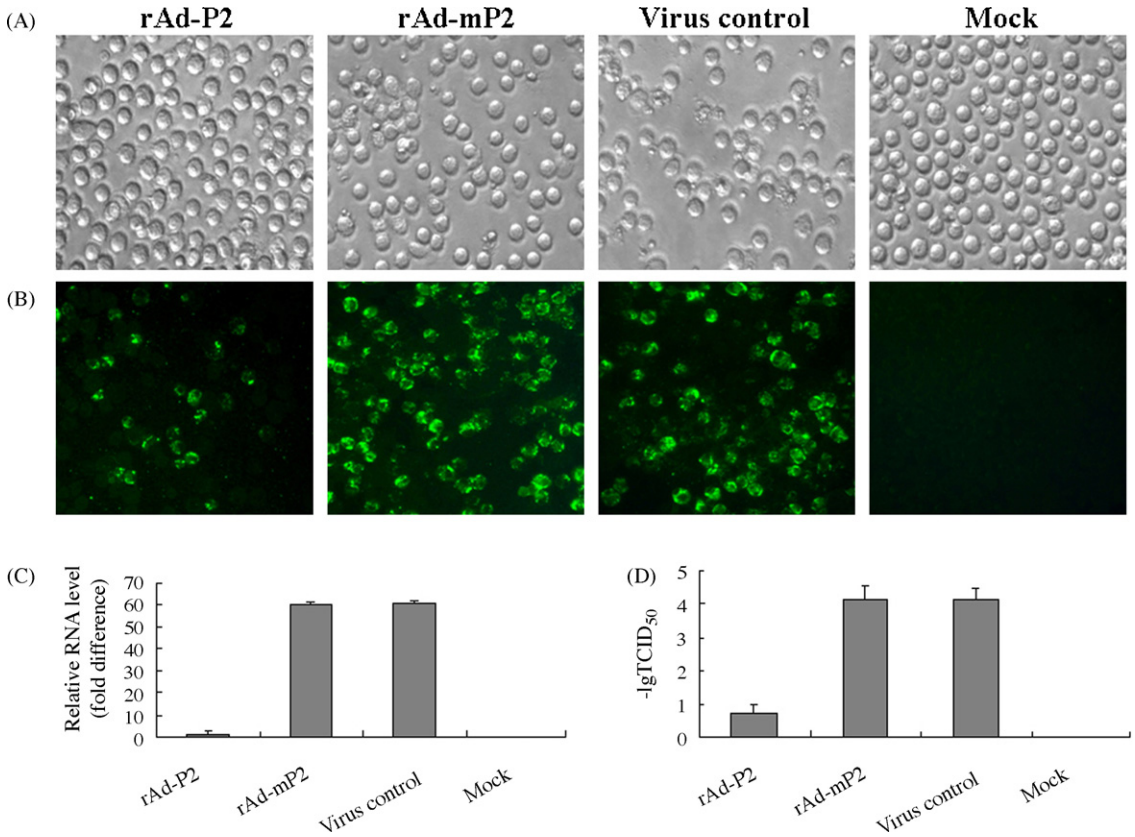

Fig. 5. Inhibition of virus replication induced by rAd-P2 in porcine alveolar macrophages. Macrophages seeded in 96-well plates were inoculated with rAd5 at 500 MOI, and challenged with PRRSV SY0608 at 10 MOI at 24 h post-inoculation. At 72 hpc, cytopathogenic effects of macrophages were observed (A), and cells were fixed and stained with anti-ORF1b antibody (Magnification, 200×) (B). In another experiment, ORF1b mRNA was detected by real-time RT-PCR. The mRNA of β-actin served as an internal reference. The level of ORF1b mRNA was normalized to the level of β-actin mRNA in the same sample (C), and virus titers in cell cultures were detected at 72 h post-challenge (D).

comparison to those in the control groups ($P < 0.05$). It indicated that the inhibition was done by shRNA expressed by rAd-P2 directly against viral genomic RNA.

3.3. rAd-P2 inhibits replication of SY0608 strain in macrophages at protein and mRNA levels

Porcine alveolar macrophages seeded in 96-well plates were inoculated with the rAd5 and challenged with PRRSV SY0608 using the same procedure as in Marc-145 cells and at 72 hpc, PRRSV was detected with different methods. The results showed that in the presence of rAd-P2 there was almost no CPE induced by PRRSV, and the frequency of fluorescence-stained cells by IFA using antibody against ORF1b were reduced significantly, when compared with those of the rAd-mP2 and PRRSV control groups. ORF1b mRNA level was decreased markedly by rAd-P2 as compared to those in other groups ($P < 0.05$). The titer of PRRSV in the rAd-P2-treated group was significantly lower than that in the rAd-mP2 and virus control groups by approximately 1000-fold ($P < 0.05$) (Fig. 5).

3.4. Inhibition of viral replication in macrophages is time- and dose-dependent

To detect the inhibition effect–time relationship, macrophages were first inoculated with the rAd5 individually, followed by PRRSV challenge at 24 hpc. At 48, 72 and 96 hpc, the cell cultures were harvested and the virus titers were measured by $CCID_{50}$. As shown in Fig. 6A, the inhibition effect induced by rAd-P2 could last for at least 96 h post-infection.

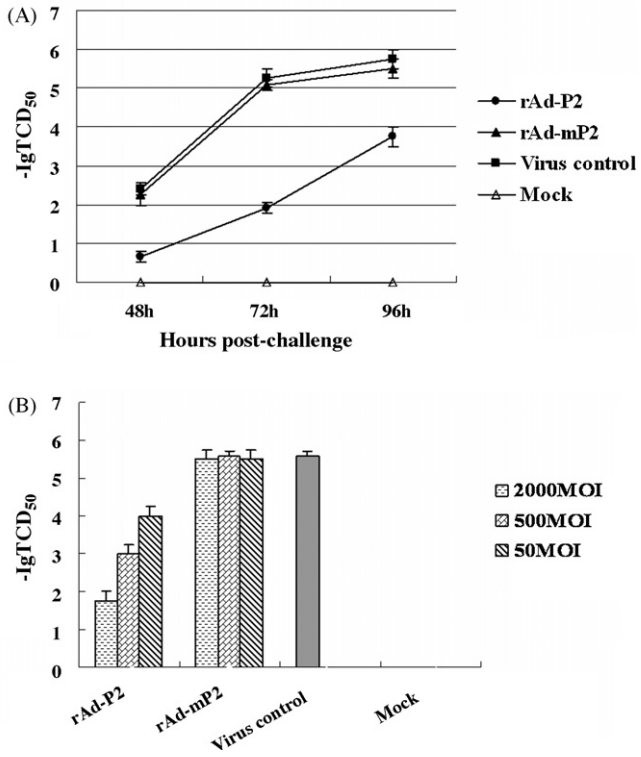

Fig. 6. The duration and dose-dependent inhibition of PRRSV replication in macrophages. Cells were infected with PRRSV at 10 MOI at 24 h after inoculation with those rAd5 at 500 MOI individually. At 48, 72 and 96 hpc, the cell cultures were harvested and the virus titers were measured by $CCID_{50}$ (A). In another test, cells were incubated with the indicated rAd5 at different MOIs for 24 h, and challenged with PRRSV SY0608 at 10 MOI and incubated for 72 h. The cell cultures were harvested and the virus yield was measured by $CCID_{50}$ (B). The data shown here was from one of three experiments.

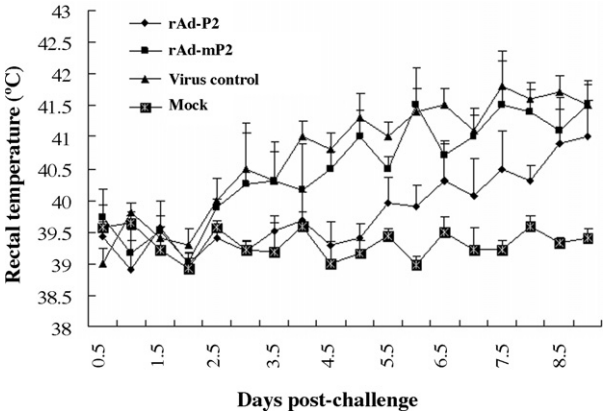

Fig. 7. Rectal temperatures of pigs in different groups at different time post-challenge. Twenty 45-days old pigs were divided randomly into four groups and five for each. Groups 1–2 were inoculated intramuscularly with rAd-P2 and rAd-mP2 at 4×10^9 $CCID_{50}$. At 24 h after inoculation groups 1–3 were challenged with 2×10^4 $CCID_{50}$ of PRRSV SY0608 intramuscularly, and group 4 was treated with PBS as a negative control. The rectal temperatures of pigs in different groups were tested every 12 h post-challenge.

To detect the dose-dependent suppression of viral replication, macrophages seeded in 24-well plates were first inoculated with the rAd5 at increasing amounts (50–2000 MOI) individually, followed by PRRSV challenge at 24 hpc. The results showed that the cells inoculated at a higher dose of rAd-P2 displayed a stronger antiviral effect at each time point, relative to the lower dose groups, as shown in Fig. 6B.

3.5. rAd-P2 decreased the pathogenicity of SY0608 strain in swine

As shown in Fig. 7, piglets in rAd-mP2 inoculated group and virus control group developed high fever (40.0–42 °C) from 3 dpc. Then the pigs displayed a range of clinical signs, including loss of appetite, lethargy, dyspnoea, red skin, eyelid oedema, lightly diarrhea and rough hair coats. However, piglets inoculated with rAd-P2 showed fever which only started from 6 dpc (40.0–41 °C) and the rectal temperature was relatively lower than that of pigs in the rAd-mP2 and control groups during the period of 3–9 dpc ($P < 0.05$). At 8 dpc, three pigs in the rAd-P2 group also showed little appetite and lethargy. The onset of disease in pigs inoculated with rAd-P2 was markedly delayed.

All pigs were examined for pathological lesions at 9 dpc. The results indicated that three of five pigs from the rAd-P2 inoculated group had minor gross lesions in lung, showing minor interstitial pneumonia, and the rest of the piglets did not show gross lung lesions. The gross lesion scores in piglets inoculated with rAd-P2 was significantly lower than those of rAd-mP2 group and virus control group ($P < 0.05$) (Table 1). No obvious microscopic lesion was

Table 1
Gross lesion score of lung of pigs in different groups after challenge.

Group	Gross lesion score of lung of different pigs[a]					Total
	No. 1	2	3	4	5	
rAd-P2	0.0	0.3	0.0	0.5	1.0	0.36 ± 0.186^{Ab}
rAd-mP2	2.8	9.3	15.0	1.8	2.0	6.18 ± 2.604^{B}
Virus control	14.7	7.8	5.3	2.4	3.0	6.64 ± 2.229
Mock	0.0	0.0	0.0	0.0	0.0	0.00 ± 0.000

[a] Gross lung pathology was assessed by using a gross pig lung lesion scoring system where each lobe of the lung was evaluated for percent pneumonia and the percent pneumonia of each lobe was added for the entire lung in a totally blind manner.

[b] A = $P < 0.05$, rAd-P2 vs. rAd-mP2 or virus control. A = $P > 0.05$, rAd-P2 vs. mock control. B = $P > 0.05$, rAd-mP2 vs. virus control.

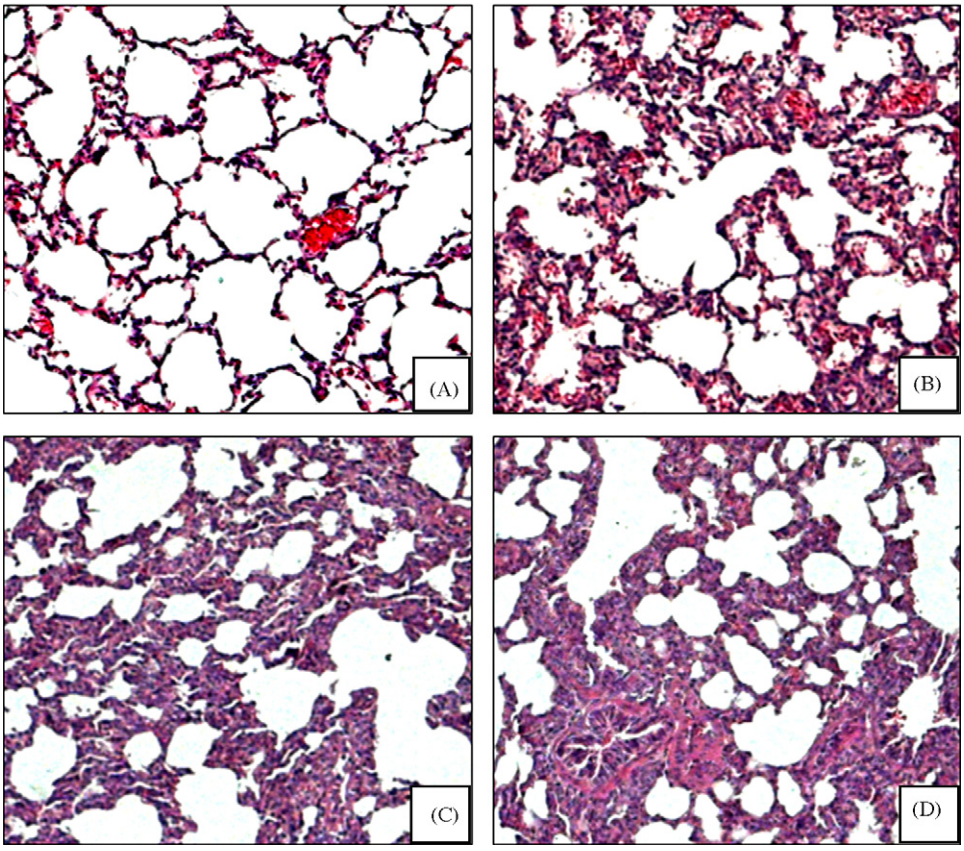

Fig. 8. Examination of histopathological lesions in the lungs of piglets in mock group (A), rAd-P2 group (B), rAd-mP2 group (C) and the virus challenge control group (D) at 9 dpc. There was no apparent interstitial pneumonitis within the lumen (thin alveolar septum) (A). Minor interstitial pneumonitis and necrotic cell debris within the lumen of a bronchiole with mild damage to the epithelium (B). Significant interstitial pneumonitis accumulated by intensive macrophage cells, normal extent of the epithelium of a large bronchus wall thickened by inflammatory cell infiltration (C and D). Hematoxylin and eosin staining (HE). Magnification, 400×.

observed in the tonsils and spleens in all piglets (data not shown). Histopathological examination of lungs showed that all the piglets in rAd-mP2 and virus control groups had clearly interstitial pneumonia, which was characterized by thickened increased alveolar walls, infiltration with intensive macrophage lymphomononuclear cells, and increased amounts of bronchiole exudates. In the rAd-P2 group only three piglets showed minor microscopic lesions of lung and the rest exhibited no apparent interstitial pneumonia (Fig. 8). All the piglets in the mock group exhibited no pathological lesions.

3.6. rAd-P2 depresses the viral load of SY0608 in serum and lung tissue of swine

For quantification of PRRSV in serum and lung tissue of swine, the same volume of serum samples were collected on 1 and 5 dpc, and the same weight of lung samples were collected on 9 dpc. The total RNA of the samples was monitored for quantitative RT-PCR, and 10-fold serial virus dilutions were used to construct the standard curve by plotting the viral titers logarithm against the measured C_T values. The results showed that the generated standard curve covered a linear range of 10^1–10^7 $CICD_{50}$/ml. The linear correlation coefficient ($R2$) between the C_T and the $CCID_{50}$/ml logarithm was repeatedly greater than 0.990. In the presence of rAd-P2, viral titers in serum were decreased significantly by approximately 100-fold when compared to the virus levels in the rAd-mP2 and PRRSV control groups on 5 dpc (Table 2). On 9 dpc, the viral titers in lungs from pigs in rAd-P2 group were also significantly lower than those in the rAd-mP2 and PRRSV control groups. There was no detectable PRRSV in the pigs inoculated with PBS (Fig. 9). It is suggested that the shRNAs expressed by rAd-P2 could induce effective inhibition of viral replication *in vivo*.

Table 2
Detection of ORF7 mRNA of PRRSV in serum of pigs with quantitative RT-PCR.

Groups	Days post-challenge	Cycle threshold (C_T)[a] (Mean ± S.D.)	$CCID_{50}$/ml (log_{10}) (Mean ± SD)
rAd-P2	1	21.65 ± 0.32	1.75 ± 0.09[Ab]
rAd-mP2	1	20.15 ± 0.23	2.21 ± 0.07[B]
Virus control	1	19.73 ± 0.10	2.34 ± 0.03
Mock	1	>35.0	0.0 ± 0.0
rAd-P2	5	20.02 ± 0.10	2.24 ± 0.09[C]
rAd-mP2	5	12.34 ± 0.15	4.60 ± 0.05[D]
Virus control	5	12.58 ± 0.10	4.54 ± 0.08
Mock	5	>35.0	0.0 ± 0.0

[a] $C_T = -3.256 \log_{10} CCID_{50} + 27.35$.
[b] A = $P < 0.05$, rAd-P2 vs. rAd-mP2 or virus control at 1 dpc. B = $P > 0.05$, rAd-mP2 vs. virus control at 1 dpc. C = $P < 0.05$, rAd-P2 vs. rAd-mP2 or virus control at 5 dpc. D = $P > 0.05$, rAd-mP2 vs. virus control at 5 dpc.

4. Discussion

RNAi is known as a powerful tool for anti-virus and gene therapy. The 21–23 nt dsRNA is competent to mediate gene-specific silencing without adverse effects to the organisms (Elbashir et al., 2001a,b; Huelsmann et al., 2006). RNAi techniques provide a way to counteract virus more specifically and directly as compared to other antiviral methods. Recently, RNAi mediated by plasmids expressing shRNA specific to PRRSV gene has been demonstrated to be able to suppress virus replication in Marc-145 cells (He et al., 2007a,b; Huang et al., 2006; Li et al., 2007a). However, the plasmid-based

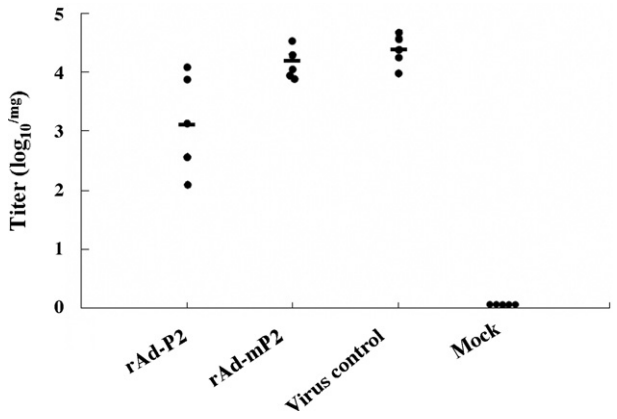

Fig. 9. The viral load in lung tissues of pigs assessed by quantitative RT-PCR for PRRSV on 9 dpc. The horizontal line corresponds to the median viral titers of the group.

strategy is not suitable to induce RNAi *in vivo*, because of the difficulty of transducing shRNA into the host cells. In contrast, viral vector expression cassettes can deliver shRNA easily into cells with high efficiency and induce steady interference with long duration. Adenovirus type 5 is tropic to respiratory epithelial cells and is competent to transfer gene into macrophages (Mayne et al., 2003; Shinozuka et al., 2000; Yang et al., 1995). It has been reported that hepatitis B (Uprichard et al., 2005), prototypic arenavirus lymphocytic choriomeningitis virus (Sánchez et al., 2005) and porcine circovirus 2 (Feng et al., 2008) and foot-and-mouth disease virus (Chen et al., 2006) could be suppressed sufficiently and effectively by adenovirus-mediated RNAi. In this study, two rAd5 expressing shRNAs targeting the ORF1b gene of the traditional PRRSV strain were constructed, and one of them was found to be effective in the presence of high virulent PRRSV SY0608 strain both in Marc-145 and PAM cells. The shRNA delivered by rAd-P2 also worked *in vivo*: the titers of PRRSV in the serum and lung tissue of swine, which were treated with rAd-P2 and then challenged with SY0608, were effectively suppressed.

ORF1ab encodes proteins with apparent replicase and polymerase activity. The ORF1ab polyprotein of PRRSV is predicted to be cleaved into at least 13 nonstructural protein products, including Nsp1α, Nsp1β, and Nsp2 to Nsp12, which are involved in virus replication and transcription and are associated with virulence (den Boon et al., 1995; Grebennikova et al., 2004; Nielsen et al., 2001; Snijder et al., 1994; van Dinten et al., 1996; Wassenaar et al., 1997). The structural proteins are translated from a nested set of 3′ coterminal subgenomic mRNAs under the control of ORF1ab proteins. shRNA specific to the ORF1b gene could suppress the gene translation and reduce the ORF1b protein in host cells as demonstrated in porcine alveolar macrophages (Fig. 5). Thus, PRRSV replication in macrophages transduced with shRNA was interrupted due to the absence of ORF1b proteins, and the cells delivered with shRNA had more chance to survive.

The results of the strong fluorescence of rAd5 GFP in Marc-145 and PAM cells showed that adenovirus could deliver gene efficiently into infected cells. There has been a report that rAd5 GFP mRNA could be detected at 8 dpc (Chen et al., 2006). However, the rAd5-expressed shRNA in this study was still incompetent to inhibit PRRSV replication completely *in vitro* and *in vivo*. The RNAi effect was reduced over time post-challenge. One simple explanation is that shRNAs delivered by adenovirus is transient as observed with transfected siRNAs, because no mutation of the ORF1b gene targeted by the shRNA was found by sequencing (data not shown). Another possible reason might be the low level of recombinant adenovirus *in vivo*, because the adenovirus is a replication-defective virus, it could not replicate *in vivo*, or it might be eradicated by the immune system. In addition, the recent reports demonstrated that adenovirus VA1 noncoding RNA can inhibit RNAi at physiological levels of expression, which result in the failure of RNAi in human cells (Andersson et al., 2005; Lu and Cullen, 2004). Some RNA virus, such as flock house virus (FHV) encoded protein, B2 (Galiana-Arnoux et al., 2006), vaccinia protein E3L and influenza protein NS1 (Li et al., 2004), could inhibit RNA silencing. Thus, the mechanism of escaping RNAi effect for PRRSV should be detected in future.

PRRSV replication was found in all organs and tissues, especially in macrophages, during acute infection. In order to evaluate the capacity of the shRNA-expressing rAd5 to suppress viral replication in swine, porcine alveolar macrophages were first used to measure the inhibition efficiency of rAd-P2. The results demonstrated that the inhibition effect induced by the ORF1b-specific shRNA remained sustained for at least 96 h. And the inhibition of viral replication was dose-dependent in PAM cells. In the animal experiments, it was found that in the presence of rAd-P2, the viral titer in serum was decreased significantly as compared to the virus levels in rAd-mP2 and PRRSV control groups on 5 dpc ($P < 0.05$). On 9 dpc, all the pigs in rAd-mP2 and virus control groups showed very clear clinical signs, but in the rAd-P2 group only three pigs showed light clinical signs. Pathological observations showed that the pneumonia in rAd-P2 inoculated pigs was significantly lighter than that of pigs inoculated with rAd-mP2 and challenge control group. It suggested that shRNAs expressed by rAd-P2 could induce effective inhibition of viral replication in pigs for several days. It may be useful for decreasing the severity of the PRRSV infection in a pig herd. Of course, the number of the experiment animals was very small, and the differences between the rAd-P2 and challenge control groups in terms of clinical signs were also minor. The protective efficiency should be detected in an experiment using larger sample sizes in the future.

PRRSV SY0608, with high pathogenicity, is a new strain of PRRSV and wildly spread in China. It has caused a severe respiratory and reproductive syndrome. In this study, the clinical signs and pathological observation in the PRRSV control group were not as severe as those reported previously (Li et al., 2007b). It might be related to the pig species or the high passage of the PRRSV SY0608 used in this study. This was also observed for another highly pathogenic PRRSV isolate JXA1 during passage of the virus in Marc-145 cells (unpublished data). Although the clinical signs, gross lesion scores of lung and pathological lesions in the rAd-mP2 group were lower than that of the PRRSV control group, there was no significant difference between those groups. Inflammatory cytokines and INF-γ may play important role in clearing virus infection (Huarte et al., 2006; Zhu et al., 2007), and hepatitis B virus replication and gene expression could be inhibited by inflammatory cytokines triggered by adenovirus infection (Cavanaugh et al., 1998). Thus, inoculation of adenovirus might activate the immune system and produce some inflammatory cytokines to decrease PRRSV replication in pigs. The production of inflammatory cytokines should be monitored in future studies.

In conclusion, two recombinant adenoviruses expressing shRNAs targeting ORF1b of PRRSV were constructed, and one could inhibit highly pathogenic PRRSV replication specifically *in vitro* and *in vivo*. The efficacy of the siRNAs should be further studied by using a large number of pigs in the future. RNAi mediated by recombinant adenovirus technology might be a potential new measure for the prophylaxis of PRRSV infection.

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