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Construction and Detection of Recombinant Adeno-Associated Virus Co-Expressing PRRSV GP5, M Proteins and shRNA

Bin Yang*, Xi Lan and Yan Qiu

State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730046, China

***Corresponding author:** Yang B, State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730046, China, Tel: +86-13919137004 ; Fax: +86-931-8340977; E-mail: beibinyang@126.com

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is a severe threat to the swine industry and has caused heavy economic losses worldwide. The currently used inactivated and attenuated virus vaccines have several shortcomings, such as unsafety and low protection rate, so it is urgent to develop a new vaccine. To explore and develop a novel vaccine against PRRSV, a bi-functional recombinant adeno-associated virus, expressing ORF5 and ORF6 proteins as well as a short interfering RNA (shRNA) against ORF7, was constructed. The shRNA against ORF7 was inserted into the sequence forward of the U6 promoter of pAAV-U6-IRES-hrGFP, and ORF5 and ORF6 were cloned into the sequence after the CMV promoter. 293T cells that were co-transfected with this vector along with pAAV-RC and pHelper produced a recombinant adeno-associated virus. 293T cells transduced with this recombinant virus expressed GP5 and M proteins, and Marc145 cells transduced with this recombinant virus suppressed the replication of PRRSV. The infective titer of the reconstructed virus was 1.9 × 1010 v.gmL as measured by the dot blotting method, and GP5 and M proteins were detected by western blot. The successful construction of rAAV-shRNA-ORF5-6 paves the way for the development of novel bi-functional vaccines against PRRSV.

Keywords: PRRSV; GP5 protein; M protein; Recombinant adeno-associated virus; shRNA; Bi-functional vaccine

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a contagious disease of swine caused by porcine reproductive and respiratory syndrome virus (PRRSV). PRRS was first reported in America in the late 1980s [1] and subsequently reported in Europe [2]. The main clinical symptoms of this highly pathogenic disease are abortion, stillbirth, breeding problems, and respiratory problems in newborns [1]. The vaccines that are currently commercially available contain inactivated or attenuated virus immunogens and can lessen the clinical severity of PRRS. However, these regimens offer limited protective efficacy and have a poor safety profile. Design of a more effective immunogen or regimen is limited by the lack of information available about the immune response to PRRSV. The protective immune mechanism elicited by current vaccines is undetermined, and the immune mechanisms responsible for clearing a natural infection of PRRSV remain unknown. Furthermore, an efficacious immunogen must elicit an immune response capable of protecting animals from genetically diverse strains of this virus.

RNA interference (RNAi) has emerged as a potent method by which the expression of specific genes can be silenced in living cells [3]. The administration of virus-sequence-targeted short interfering RNA (siRNA) with a length of 21–33 nucleotides has inhibited viral replication effects in mammalian cells and animals [4]. Short hairpin RNA (shRNA) can also downregulate the expression of viral genes and protect animals from virus challenge through binding to virus-specific mRNA [5]. RNAi has already been employed as a mechanism of therapeutic gene silencing and anti-viral therapy.

The PRRSV genes ORF1a and ORF1b encode nonstructural proteins, while ORFs 2–7 encode structural proteins [6]. ORF5 encodes GP5, a trans membrane glycoprotein present on the surface of the viral particle. Antibodies targeting epitopes in the GP5 ectodomain can neutralize PRRSV and are elicited during natural infection by PRRSV. Although the genetic diversity in circulating PRRSV GP5 proteins is high [7,8], recombinant GP5 is an integral component of several efficacious vaccines against PRRSV [9,10]. ORF6 encodes a matrix protein (M), which is the most highly conserved PRRSV protein [11]. The GP5 and M proteins are crucial for the PRRSV life cycle [12]. GP5 and M proteins are also immunogenic and act as the targets of protective antiviral immune responses. ORF7 encodes the protein N, which encapsulates the viral RNA genome [13]. The N protein is highly antigenic and is mainly used for the diagnosis and genotyping of PRRSV [14,15]. Adeno-associated virus (AAV) has been widely used as a gene therapy vector due to its low immunogenicity and ability to infect both dividing and non-dividing cells [16]. We have constructed a recombinant AAV co-expressing ORF5 and ORF6 as well as shRNA directed against PRRSV ORF7. With this recombinant virus, we intend to equip the vaccinated host with the ability to inhibit ORF7 expression by RNAi while simultaneously provoking GP5 and M protein-directed immune responses.

Materials and Methods

Materials

293T and Marc145 cells were acquired from the Key Laboratory of Epizootic Diseases of Grazing Animals of the Ministry of Agriculture, Lanzhou Veterinary Research Institute, Lanzhou, China. RNA extraction kits were purchased from Qiagen Company (Germany), a pAAV Helper-Free system was purchased from Stratagene, and

plasmid extraction kits, gel extraction kits, reverse transcriptase, Premix Taq DNA polymerase, T4 DNA ligase, cloning vector pSimple 19-T, and ligation kits were all bought from Takara (Dalian, China). Restriction endonucleases BamHI, EcoRI, XhoI, XhaI, and Age I was purchased from New England Biolabs (UK) Ltd (UK). Fetal bovine serum (FBS) and high glucose DMEM were purchased from Gibco (USA), the transfection reagent Lipofectamine™ 2000 was purchased from Invitrogen (USA), and digoxin DNA labeling and detecting kits were purchased from Roche (Germany). Hyper-immune serum against PRRSV was bought from the China Institute of Veterinary Drugs Control. Horse radish peroxidase-labeled rabbit anti-pig IgG was purchased from Beijing Aobosen Biotechnology Company, and GoTaq® 2-Step RT-PCR System kit was purchased from Promega. All primers were synthesized by Shenggong (Shanghai, China).

Construction of adeno-associated virus co-expressing PRRSV shRNA and ORF5-6 genes

Construction and identification of pAAV-U6-IRES-hrGFP-shRNA

Primer design, synthesis, and PRRSV-shRNA preparation

Primers were designed according to the genomic sequence of PRRSV JXA1 (Gene Bank accession no. EF112445.1) and the target sequence of ORF7 (GATGACGTCAGGCATCACT (14979–14997)) screened by He et al. [17], as follows: SH-A:5′-CCGGTGATGACGTCAGGCATCACTTTCAAGACGAGTGATGCC TGACGTCATCTTTTTTT-3′ (underlined sequence is a recognition site for AgeI); SH-B: 5′-CTAGAAAAAAAGATGACGTCAGGCATCACTCGTCTTGAAAGT GATGCCTGACGTCATCA-3′ (underlined sequence is a recognition site for Xba I). Nucleotides for each primer were randomly rearranged as a negative control, with the same restriction sites and hairpin loop sequences. Primers for use as a negative control were as follows: N-SH-A: 5′-CCGGTTAGTAACTCGCAGCTCAGGTTCAAGACGCCTGAGCTG CGAGTTACTATTTTTTT-3′N-SH-B: 5′CTAGAAAAAAATAGTAACTCGCAGCTCAGGCGTCTTGAACC TGAGCTGCGAGTTACTAA-3′. Control primers and 2 μL of the synthesized SH fragments, SH-A and SH-B, were mixed with 16 μL of annealing buffer, incubated at 94°C for 10 min, gradually cooled to room temperature, and stored at 4°C for later use at a 100-fold dilution.

Ligation of pAAV-U6-IRES-hrGFP and the SH fragment

Xba I and Age I were used to digest pAAV-U6-IRES-hrGFP and the resulting product was subjected to 1.5% agarose gel electrophoresis. The larger fragment was recovered from the gel, ligated with the diluted SH fragment (described above) by T4 DNA ligase, and transferred into competent DH5α cells. Positive colonies were selected for DNA sequencing after incubation in liquid media. The positive plasmid was named pAAV-U6-IRES-hrGFP-shRNA, and the control plasmid was named N-pAAV-U6-IRES-hrGFP.

Construction of pAAV-U6-IRES-hrGFP-shRNA-ORF5-6

Cloning of ORF5 and ORF6

Using DNAStar and Oligo 6.0, ORF5 and ORF6 gene-specific primers were designed with endonuclease recognition sites (underlined) at the 5′ end of each primer based on the PRRSV JXA1 genomic sequence and the sequences and multiple cloning sites of pAAV-U6-IRES-hrGFP as follows: F51: 5′-ATAGGATCCaccatgttggggaagtgcttg-3′ (BamH I); F52: 5'-GCGAATTCAAGAAGGTCAAAATTCAACAGctagagacgaccccatag-3' (EcoRI, Bold capitalized characters represent the sequences for 2A); F61: 5′-ATAGAATTCatggggtcgtctctagacga-3′ (EcoRI); F62: 5′-AGCctcgagttatttggcatatttaacaagg-3′ (XhoI) (Capital letters represent the sequence of the enzyme and the protective base group).

The total PRRSV JXA1 RNA was extracted, and then ORF5 and ORF6 were amplified by RT-PCR with the primers described above. Fragments of 530 bp and 600 bp were recovered, ligated into T vector, and transferred into competent DH5α cells. Positive colonies were selected for DNA sequencing after incubation in liquid media.

Construction and identification of pAAV-U6-IRES-hrGFP-shRNA-ORF5-6

BamH I and EcoR I were used to digest pSimple 19-T-ORF5, and EcoRI and XhoI were used to digest pSimple 19-T-ORF6. The resulting ORF5 and ORF6 fragments were isolated by agarose gel electrophoresis. BamH I and Xho I were used to digest pAAV-U6-IRES-hrGFP-shRNA, and the resulting product was subjected to agarose gel electrophoresis. The larger fragment with sticky ends was recovered for use in later experiments. The recovered ORF5, ORF6, and pAAV-U6-IRES-hrGFP-shRNA were combined at a ratio of 1:1:1, ligated at 16°C overnight, and transferred into competent JM109 Escherichia coli cells. Positive colonies were identified by PCR and double digestion, after which they were sequenced and named pAAV-U6-IRES-hrGFP-shRNA-ORF5-6.

Preparation of recombinant adeno-associated virus (rAAV)

Two days prior to packaging, 293T cells were cultured in DMEM containing 10% FBS and seeded at the density of 3 × 106 cells per 100-mm dish. When the cells reached 60% confluence, they were co-transfected by calcium phosphate transfection with either pAAV-U6-IRES-hrGFP-shRNA-ORF5-6 or a control plasmid, along with the packaging plasmid pAAV-RC and helper plasmid pHelper (10 μL each). Transfected cells were cultured at 37 °C with 5% CO2. DMEM containing 10% FBS was replaced 24 h after transfection, and the cells were cultured for a further 68–72 h. Cellular morphology was observed with an inverted microscope every 24 h. The cells and supernatant were then harvested and subjected to four rounds of −70°C/37°C freeze-thaw cycles. After centrifugation for 10 min at room temperature at 10000 ´g, supernatant containing virus rAAV-shRNA-ORF5-6 was obtained and stored at −72°C. Negative controls rAAV, N-rAAV, and rAAV-shRNA were prepared the same way.

Isolation and purification of rAAV

According to a previously described method (Wu X. et al. 2000), 20 mL of transfected cells were collected and thoroughly mixed with 2 mL of chloroform. Sodium chloride was added to produce a final concentration of 1 M. After centrifugation, the supernatant was collected and added to PEG8000 to a final concentration of 10% (w/v), followed by centrifugation. The supernatant was discarded, and the precipitate was dissolved in 0.5 mL of phosphate-buffered saline. DNase was added to a final concentration of 1 μg/mL, and digestion was performed at room temperature for 30 min. After digestion, an equal volume of chloroform was added, and the purified virus rAAV was obtained in the supernatant after centrifugation.

Virus titration of rAAV

A digoxin-labeled CMV probe was used for dot blotting to test the titer of rAAV. CMV-specific primers were designed, using pAAV-U6-IRES-hrGFP plasmid DNA as a template: upstream primer: 5′-CTTATATAGACCTCCCACC-3′; downstream primer: 5′-GACGTCAATGACGGTAAAT-3′. Probes were labeled by PCR, and then pAAV-U6-IRES-hrGFP was quantifiedby detecting the concentration of the DNA plasmid. Purified rAAV and the standard plasmid (10 μL each) were boiled for 10 min and immediately chilled in ice. They were then used in a two-fold serial dilution with TE buffer and spotted on a membrane, which was baked at 120°C for 30 min and pre-hybridized at 68°C overnight. After membrane washing and development, the reaction was stopped. Copies of CMV were evaluated through comparing the signal intensity of rAAV and standard (v.g.). The rAAV titer was calculated by using the following formula: rAAV titer=copies of pAAV-U6-IRES-hrGFP × dilution ratio of standard plasmids with the same signal intensity to that of rAAV.

rAAV-shRNA-ORF5-6 infection of 293T cells and detection of E and M proteins

293T cells were seeded in six-well plates at a density of 3 × 105 cells per well and cultured at 37°C with 5% CO2. After 12 h, the media was discarded and the cells were gently washed twice with DMEM in the absence of FBS. After a ten-fold serial dilution (from 109 to 105 viral particles), 500 μL of rAAV-shRNA-ORF5-6 virus was added to each well. Virus absorption was promoted with gentle shaking every 20 min for 1 h, after which regular media was added to each well with the addition of 10–50 mmol/L sodium butyrate. After culture at 37 °C with 5% CO2 for 48 h, the optimal multiplicity of infection (v.g./mL) was determined by counting green fluorescent cells under a fluorescence microscope. Six wells of 293T cells were infected with 107 v.g./mL of rAAV-U6-IRES-hrGFP-shRNA-ORF5-6 and, 72 h later, the cells were digested with 0.1% trypsin and harvested. The cells were then treated with lysis buffer for 1 h and subjected to centrifugation at 10,000 ´g for 10 min. The resulting supernatant was collected, subjected to 12% SDS-PAGE with protein loading buffer, transferred onto a NEPC membrane, and blocked with 5% non-fat milk powder overnight. After three 5-min washes with PBST, PRRSV hyper-immune serum (diluted 1:200) was added, and the membrane was blocked at 4°C overnight. After three 5-min washes with PBST, horse radish peroxidase-labelled rabbit anti-pig IgG (diluted 1:3000) was added for additional 40-min incubation. After three more 5-min washes with PBST, ECL was used to develop the membrane.

Analysis of RNAi inhibition of PRRSV replication

To assess the RNAi inhibition effect of the recombinant construct rAAV-U6-IRES-hrGFP-shRNA on PRRSV replication in Marc145 cells, these cells were seeded in six-well plates at a density of 5 × 105 cells per well and cultured at 37°C with 5% CO2 for 24 h. When the cells grew to 70–80% confluence, they were infected with rAAV-U6-IRES-hrGFP-shRNA or N-rAAV-U6-IRES-hrGFP-shRNA at the concentration with the optimal infection efficiency. After 1 h of virus absorption, regular culture media was added, and the cells were cultured at 37°C with 5% CO2. The media was discarded after 24 h, and 200 μL of 100 TCID50 PRRSV was added. After 1 h of absorption, the cells were cultured in maintenance media. Pathological changes were observed within 72 h by electron microscopy.

Results

Construction of the recombinant vector, pAAV-U6-IRES-hrGFP-shRNA-ORF

The pAAV-U6-IRES-hrGFP-shRNA-ORF vector was constructed according to the plan illustrated in Figure 1. ORF5 and ORF6 were cloned by PCR amplification using primers specific to ORF5 or ORF6. The amplified fragments were sequenced and verified according to the PRRSV JXA1 ORF5 and ORF6 sequences in GenBank (accession number JQ804986.1). We found that ORF5 shares 99.3% sequence similarity to PRRSV JXA1, while ORF6 shares 99.4% with PRRSV JXA1. The amino acid sequence was identical to that of PRRSV JXA1. Next, the SH fragment was ligated into pAAV-U6-IRES-hrGFP and transformed into E. coli, after which the plasmid was extracted and subjected to sequencing to confirm that SH was successfully inserted into pAAV-U6-IRES-hrGFP. ORF5 and ORF6 were then ligated into pAAV-U6-IRES-hrGFP-SH and transformed into E. coli. We found by PCR identification and enzyme digestion that ORF5 and ORF6 were ligated into pAAV-U6-IRES-hrGFP-SH as expected, and a recombinant vector co-expressing pAAV-U6-IRES-hrGFP-shRNA-ORF5-6 was successfully constructed (Figure 2).

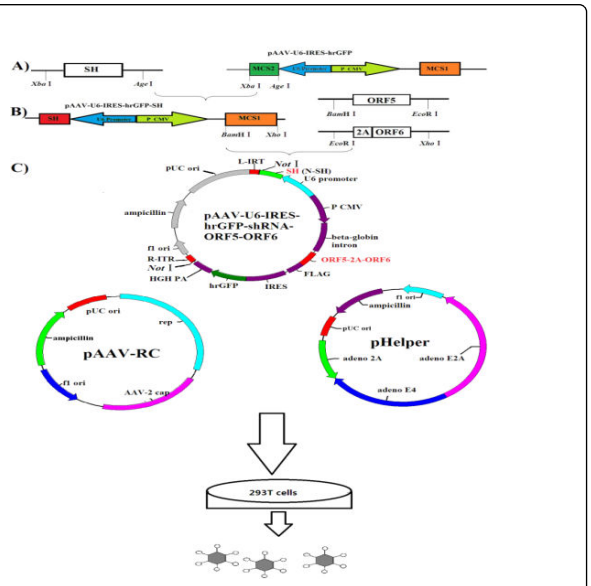

Figure 1: Schematic of the vector construction for rAAV. (A) SH was inserted into pAAV-U6-IRES-hrGFP at the CMS2 site. (B) ORF5 and ORF6 were inserted into pAAV-U6-IRES-hrGFP-SH at the CMS1 site. (C) pAAV-U6-IRES-hrGFP-shRNA-ORF5-6 and helper plasmids pHelper and pAAV-RC were co-transfected into 293T cells to produce rAAV-shRNA-ORF5-6.

Co-transfection of the recombinant vector and packaging of the recombinant AAV

To assess the occurrence of viral packaging after transfection, we examined the appearance of the cells co-transfected with the recombinant vector and two helper plasmids, pAAV-RC and pHelper. During viral packaging in 293T cells, the color of the media gradually turned from red to yellow. Cells co-transfected with this vector, pAAV-RC, and pHelper also rounded up and detached from the flask,

suggesting successful viral packaging was taking place in these cells (Figure 3).

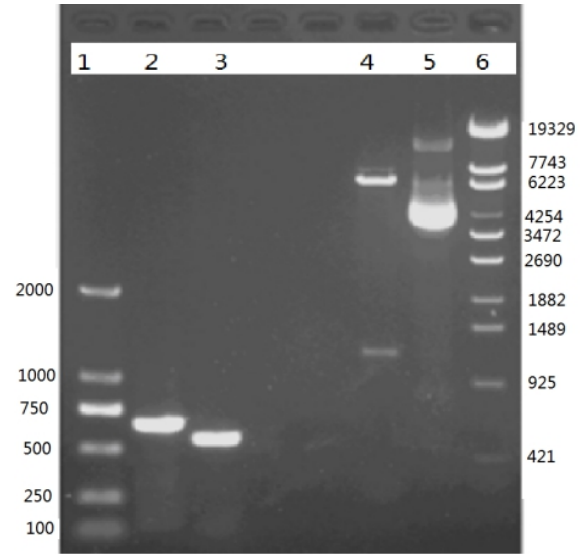

Figure 2: Identification of pAAV-U6-IRES-hrGFP-shRNA-ORF5-6 by double digestion and PCR. To confirm successful construction of pAAV-U6-IRES-hrGFP-shRNA -ORF5-6, this plasmid was digested with BamH I and Xho I and then amplified by PCR; the resulting products were subjected to agarose gel electrophoresis along with undigested plasmid. Lane 1: DNA 2000 Marker; lane 2: PCR product of ORF5; lane 3:PCR product of ORF6 ; lane 4: pAAV-U6-IRES-hrGFP-shRNA-ORF5-6 (digested with BamH I and Xho I); lane 5: pAAV-U6-IRES-hrGFP-shRNA-ORF5-6 (without digestion); lane 6: λ-Eco T14 I digested DNA Marker.

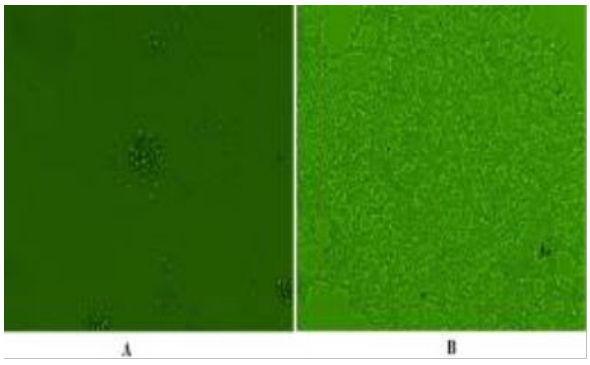

Figure 3: Representative images of co-transfected or untransfected 293T cells 72 h post-transfection. The cells were viewed with an inverted microscopeA: 293T cells were co-transfected with pAAV-U6-IRES-hrGFP-shRNA-ORF5-6 and two helper plasmids, pAAV-RC and pHelper, the representative images of rAAV at 72 h post-cotransfection.Co-transfected 293T cells rounded up and detached from the flask, and rAAV viral paticles could be seen in the picture; B : 293T cells untransfected (B).

Isolation and purification of virus

To isolate the virus, we subjected the transfected 293T cells to several cycles of freeze-thaw treatment and then purified the resulting rAAV as described in the Methods. To confirm the identity and purity of the product, 0.5 mL of it was subjected to SDS-PAGE. The rAAV isolates produced three clear bands on the gel, with molecular weights of 87 Ku, 72 Ku, and 62 Ku, representing the envelope protein of AAV (Figure 4).

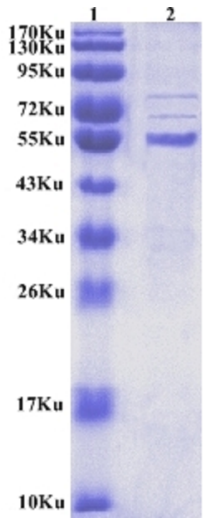

Figure 4: SDS-PAGE of purified rAAV, staining by Commassie blue. Purified rAAV was subjected to SDS-PAGE and the resulting gel was stained with Comassie blue. Lane 1: Predyed 10-170 Ku Protein Marker. Lane 2: Purified expressed recombinant rAAV-shRNA-ORF5-6. The molecular weights of the three bands are 87 Ku, 72 Ku, and 62 Ku.

Titration of rAAV

To determine the titer of the rAAV, we performed dot-blotting assays. We used pAAV-U6-IRES-hrGFP, a plasmid of 6.1 Kb, as the standard plasmid at an initial concentration of 7.6 × 1010 copies/mL. TE buffer was used to create several different dilutions of both the standard plasmid and the rAAV. The signal intensity of rAAV in the dot blot was similar to that of the forth dilution of the standard plasmid, indicating that the titer of rAAV was roughly 1.9×1010 v.g./mL, a concentration which is appropriate for animal inoculation (Figure 5).

Detection of E and M protein expression by western blot

To assess the effect of infection with our recombinant virus, 48 h after infecting 293 T cells with rAAV-U6-IRES-hrGFP-shRNA-ORF5-6, we observed them under a fluorescence microscope (Figure 6). The highest level of infection was achieved when infection was performed with rAAV-U6-IRES-hrGFP-shRNA-ORF5-6 at a concentration of 106 v.g./mL. We then performed a western blot on the cell lysates of cells infected under these conditions. We detected the expression of E and M proteins with their expected molecular weights of 18 Ku and 25 Ku, respectively (Figure 7).

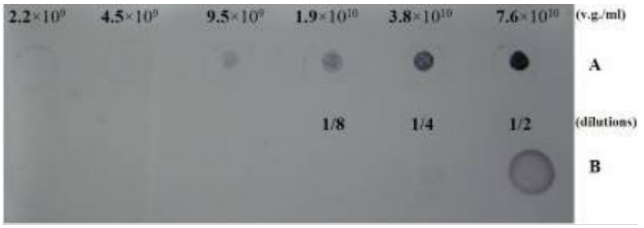

Figure 5: Determination of rAAV-shRNA-ORF5-6 titer by dot-blotting. Dot-blotting was performed to assess the titer of purified recombinant rAAV-shRNA-ORF5-6 by a digoxin-labelled CMV probe .Copies of CMV were evaluated through comparing the signal intensity of rAAV and standard (v.g.). The rAAV titer was calculated by using the following formula: rAAV titer = copies of pAAV-U6-IRES-hrGFP × dilution ratio of standard plasmids with the same signal intensity to that of rAAV.(A) a two-fold Serial dilution of the standard plasmid, pAAV-U6-IRES-hrGFP,. (B) a two-fold Serial dilution of rAAV-shRNA-ORF5-6.

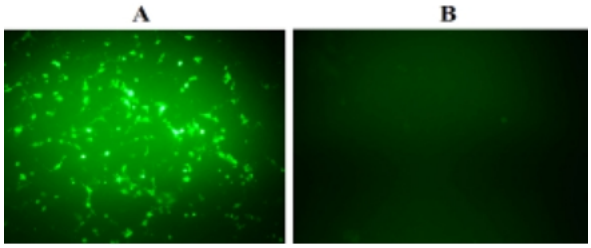

Figure 6: Optimization of rAAV-shRNA-ORF5-6 infection efficiency in 293 T cells. 293 T cells were infected with rAAV-shRNA-ORF5-6 or left uninfected. Fluorescence images taken 48 h after infection show the best transfection efficiency group (A) and the uninfected control group (B). The cells were viewed under a fluorescence microscope.

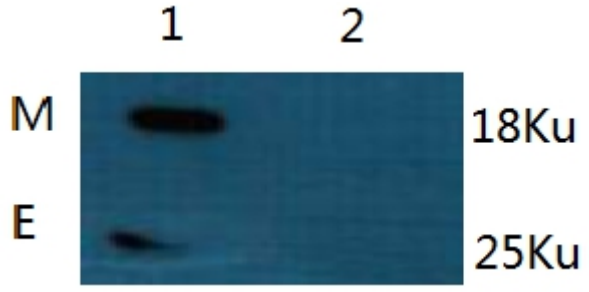

Figure 7: Western blot analysis of E and M proteins expressed in 293T cells. 293T cells were infected with rAAV-shRNA-ORF5-6 or left uninfected, and lysates of these cells were used in a western blot to assess the expression of E and M proteins. Lane 1: infected group; lane 2: uninfected control group.

Analysis of RNAi effect against PRRSV amplification

To assess the RNAi effect of the rAAV against PRRSV amplification, we infected Marc145 cells with rAAV-shRNA or N-rAAV before challenging them with PRRSV, as described in the Methods. Cells infected with rAAV-shRNA before the PRRSV challenge showed a higher survival rate than that of the cells infected with the control N-rAAV (Figure 8) [18]. The inhibition efficiencies of the rAAV-shRNA and N-rAAV treatments were calculated as 36.3% and 15.0%, respectively , indicating that rAAV-shRNA inhibited PRRSV proliferation.

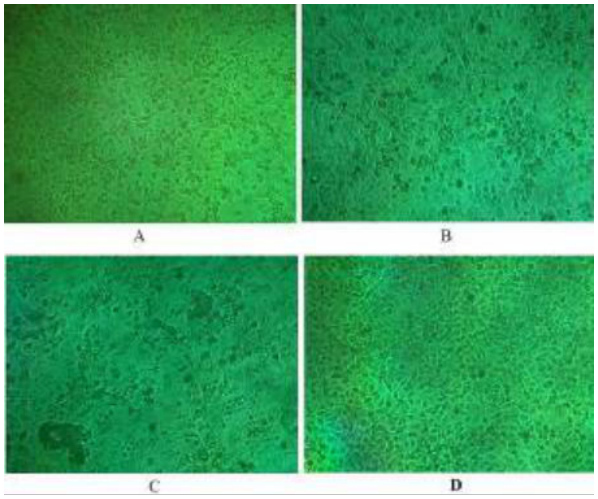

Figure 8: Assessment of RNAi inhibition of PRRSV replication in Marc145 cells.Representative images are shown with an inverted microscope(A) Marc145 cells infected with rAAV-shRNA-ORF5-6 and challenged it with100TCID50 PRRSV 72h post-infection. (B) Marc145 cells infected with N-rAAV control and challenged it with PRRSV 72h post-infection.(C) Marc145 cells challenged with PRRSV. (D) Marc145 cells control.

Discussion

AAV replication is dependent upon other viruses [19]. Fourteen serotypes of AAV have been described, the most regular of which is type 2. The first clinical use of rAAV was as a gene therapy vector in the treatment of hemophilia [20]. This recombinant virus possesses many qualities that make it a popular choice for gene therapy. Clinical experiments indicate that rAAV elicits low toxicity, achieves prolonged and high-efficiency gene expression and is capable of infecting a variety of cell types [21]. Additionally, rAAV has facilitated successful gene therapy of several chronic and intractable diseases. For example, Jiang et al. [22] successfully treated type A hemophilia in dogs with types 5, 6, and 8 AAV vectors expressing blood coagulation factor. The cell-specificity of AAV subtypes can be exploited to direct gene therapy to a particular cell type. Bell et al. demonstrated that AAV8 infects hepatic central veins efficiently, but infects hepatic portal veins with a greatly reduced efficacy. Furthermore, rAAV vectors are also widely used in anti-tumor therapy. Lv et al. expressed DR5 directed antibodies in rAAV to inhibit the growth of tumor cells [23].

Although several PRRSV vaccines are commercially available, the PRRS pandemic remains of great economic importance. These

inactivated and attenuated virus vaccines are unable to safely elicit effective protective immunity [24]. Although attenuated virus vaccines provide some protection, their potential toxicity is worrisome [25]. Further research is necessary to understand the pathogenicity of and immune response to PRRSV. This data will facilitate the rational design of novel PRRSV immunogens.

Here, we developed a novel recombinant viral vector intended to elicit immunity to PRRS while also conferring the capability to inhibit PRRSV replication. A PRRSV-directed RNAi sequence was inserted into an ORF5- and ORF6-encoding AAV vector. Marc145 cells infected with the resultant viral vector sustained less PRRSV replication than controls. The shearing function of polypeptide 2A encoded by the 2A region of foot-and-mouth disease virus allows the independent expression of two genes with high efficiency. [25] Furthermore, polypeptide 2A can also reinforce the translation of virus proteins through the induction of the voluntary binding of cytokines with virus RNA. Our western blot results confirmed that the insertion of the 2A sequence in our vector resulted in the independent expression of both GP5 and M proteins.

After purification, rAAV showed a titer of 1.9´1010 v.g./mL, a concentration that is appropriate for animal administration [26]. Thus, it will be usable in future experiments to assess the safety and immunogenicity of this adeno-associated bi-functional vaccine in vivo. In summary, we have successfully constructed an adeno-associated virus, AAV-shRNA-ORF5-6, which provides a foundation for developing effective bi-functional vaccines against PRRSV.

References

1. Cho JG, Dee SA (2006) Porcine reproductive and respiratory syndrome virus. Theriogenology 66: 655-662.
2. Office International Epizootics (OIE) (1992) World Animal Health 1991. Animal Health Status and Disease Control Methods (Part One: Reports) 2: 126.
3. Yu JY, DeRuiter SL, Turner DL (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc Natl Acad Sci USA 99: 6047-6052.
4. Jacque JM, Triques K, Stevenson M (2002) Modulation of HIV-1 replication by RNA interference. Nature 418: 435-438.
5. Wang H, Wu J, Liu X, He H, Ding F, et al. (2012) Identification of short hairpin RNA targeting foot-and-mouth disease virus with transgenic bovine fetal epithelium cells. PLoS One 7: e42356.
6. Grebennikova TV, Clouser DF, Vorwald AC et al (2004) Genomic characterization of virulent, attenuated, and revertant passages of a North American porcine reproductive and respiratory syndrome virus strain. Virology 321: 383-390.
7. Mardassi H, Mounir S, Dea S (1995) Molecular analysis of the ORFs 3 to 7 of porcine reproductive and respiratory syndrome virus, Quebec reference strain. Arch Virol 140: 1405-1418.
8. Jiang WC, Zhao MD (2009) Construction of recombinant canine adenovirus type-1 expressing the GP5 of porcine reproductive and respiratory syndrome virus(PRRSV) and its immunological property in pigs. Chin J Vet Sci 29: 1242-1250.
9. Qiu HJ, Tian ZJ, Tong GZ, et al (2005) Protective immunity induced by a recombinant pseudorabies virus expressing the GP5 of porcine reproductive and respiratory syndrome virus in piglets. Vet Immunol Immunopathol 106: 309-319.
10. Xue Q, Zhao YG, Zhou YJ (2004) Immune responses of swine following DNA immunization with plasmids encoding porcine reproductive and respiratory syndrome virus ORFs 5 and 7, and porcine IL-2 and IFNgamma. Vet Immunol Immunopathol 102: 291-298.
11. Ostrowski M, Galeota JA, Jar AM, Platt KB, Osorio FA, et al. (2002) Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J Virol 76: 4241-4250.
12. Plagemann PG (2004) GP5 ectodomain epitope of porcine reproductive and respiratory syndrome virus, strain Lelystad virus. Virus Res 102: 225-230.
13. Jiang W, Jiang P, Li Y (2006) Recombinant adenovirus expressing GP5 and M fusion proteins of porcine reproductive and respiratory syndrome virus induce both humoral and cell-mediated immune responses in mice. Vet Immunol Immunopathol 113:169-180.
14. Spilman MS, Welbon C, Nelson E (2009) Cryo-electron tomography of porcine reproductive and respiratory syndrome virus: organization of the nucleocapsid. J Gen Virol 90: 527-535.
15. Vimolmangkang S, Gasic K, Soria-Guerra R, Rosales-Mendoza S, Moreno-Fierros L, et al. (2012) Expression of the nucleocapsid protein of porcine reproductive and respiratory syndrome virus in soybean seed yields an immunogenic antigenic protein. Planta 235: 513-522.
16. Grimm D, Kay MA (2003) From virus evolution to vector revolution: use of naturallyoccurring serotypes of adeno-associated virus (AAV) as novel vectors for human gene therapy. Curr Gene Ther 3: 281-304.
17. He YX, Hua RH, Zhou YJ (2007) Rapid assessment of anti-PRRSV siRNA efficacy using PCR-derived POI shRNA cassettes. Chin. J. Prev. Vet. Med 376-381.
18. Wu XB, Dong XY, Wu ZJ (2000) A fast and effective way of separated and purfied recombinant adenoassociated virus vectors 45: 2017-2022.
19. Kienle E, Senís E, Börner K, Niopek D, Wiedtke E, et al. (2012) Engineering and evolution of synthetic adeno-associated virus (AAV) gene therapy vectors via DNA family shuffling. J Vis Exp.
20. Snyder RO, Miao CH, Patijn GA, Spratt SK, Danos O, et al. (1997) Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nat Genet 16: 270-276.
21. Adeno-associated virus expression systems for gene transfer. Curr Opin Biotechnol 9:470-475.
22. Jiang H, Lillicrap D, Patarroyo-White S et al (2006) Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs. Blood 108:107-115
23. Lv F, Qiu Y, Zhang Y, Liu S, Shi J, et al. (2011) Adeno-associated virus-mediated anti-DR5 chimeric antibody expression suppresses human tumor growth in nude mice. Cancer Lett 302: 119-127.
24. Bell P, Wang L, Gao G, Haskins ME, Tarantal AF, et al. (2011) Inverse zonation of hepatocyte transduction with AAV vectors between mice and non-human primates. Mol Genet Metab 104: 395-403.
25. Halpin C, Cooke SE, Barakate A, El Amrani A, Ryan MD (1999) Self-processing 2A-polyproteins-asy stem for co-ordinate expression of multiple proteins in transgenic plants. Plant J 17: 453-459.
26. Meng X J (2000) Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet Microbiol 74: 309-329.