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Suppression of duck hepatitis A virus Type 1 replication by lentivirus-mediated RNA-dependent RNA polymerase (RdRp) gene-specific siRNA

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To explore the application of RNAi technology for the control of duck hepatitis A virus type 1 (DHAV-1). The RNA-dependent RNA polymerase (RdRp) gene was amplified by PCR. pEGFP-RdRp was constructed for fusion expression of EGFP-RdRp protein. According to the sequence of RdRp, three gene-specific siRNAs were designed and the corresponding shRNA was inserted into pmiRZipΔ to construct pRdRp-shRNA. pRdRp-shRNA and pEGFP-RdRp were co-transfected into HEK293T cells for effective siRNA screening using fluorescent microscopy, flow cytometry, and quantitative fluorescence PCR. More effective siRNA was selected for further study using lentivirus vector pmiRZip. After demonstration of successful preparation of recombinant lentivirus, the suppressing effect was determined by calculating the 50% tissue culture infection dose ($TCID_{50}$) and RdRp gene expression of DHAV-1 in the duck embryo fibroblast (DEF) cells infected with recombinant lentivirus followed by DHAV-1 infection. The results indicated that all of the three siRNAs could suppress the RdRp gene expression, and the shRNA2 containing GDD motif had the highest efficiency. The recombinant lentivirus corresponding to shRNA2 reduced the $TCID_{50}$ of DHAV-1 by 6.2 l g and the RdRp gene expression by 89.6%, with the suppressing effect continued for at least 120 h. This work provides a new idea for the clinical control of duck virus hepatitis.

Key words: duck virus hepatitis, GDD motif, RNA-dependent RNA polymerase (RdRp), RNAi, siRNA.

INTRODUCTION

Duck hepatitis A virus type 1 (DHAV-1) is currently the primary pathogen causing duck virus hepatitis and can cause the acute hepatitis in ducklings, characterized by rapid propagation and the mortality up to 100%. Hence, duck virus hepatitis is classified as class B animal epidemics by the World Organization for Animal Health

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(OIE). The high incidence of virus hepatitis in ducklings inoculated with DHAV-1 vaccine in recent years indicates the variation of DHAV strains or reversion of virulence of traditional attenuated vaccine strains (Gao et al., 2012; Lambeth et al., 2006). The complete genome sequence of DHAV-1 was first reported in 2006 (Kim et al., 2006). Its genome is a single-stranded positive-sense RNA, encoding 2,249 amino acids and the protein 5′-UTR-VP0-VP3-VP1-2A1-2A2-2A3-2B-2C-3A-3B-3C-3D-3′ (Tseng et al., 2007; Li et al., 2013). 3D protein is an RNA-dependent RNA polymerase (RdRp), and plays a critical role in RNA replication. It can specifically recognize viral RNA, participates in the selection of RNA template and initiation site for RNA synthesis, maintains the extension of RNA synthesis, coordinates RNA synthesis procedure and regulates virus variation (Snijder et al., 2006). As an ancient and conservative defense mechanism, RNA interference (RNAi) has many unique advantages with wide applications in the control of animal virus diseases. Gene-specific siRNA can completely or partially interfere with the replication of several viruses, including chicken influenza virus (Ge et al., 2003), immunodeficiency virus (Boden et al., 2004), papillomavirus (Jiang and Milne, 2002), foot-and-mouth disease virus (Luo et al., 2011; Xu et al., 2012), bovine viral diarrhea virus (Lambeth et al., 2006; Ni et al., 2012), porcine reproductive and respiratory syndrome virus (Bao et al., 2010; Huang et al., 2006), porcine circovirus (Wang et al., 2008), and infectious bursal disease virus (Gao et al., 2008). However, there is still no application of RNAi in duck virus hepatitis. This study screened the siRNAs that can effectively silence the RdRp gene of DHAV-1, and used lentivirus vector to express siRNA to suppress DHAV-1 replication, which is of great significance to control duck virus hepatitis.

MATERIALS AND METHODS

Reagents and instruments

Restriction enzyme, T4 DNA ligase, RevertAid M-MuLV reverse transcriptase, Taq DNA polymerase, 1 kb DNA Ladder, RNase inhibitor and Trizol were purchased from Thermo Fisher Scientific; Dulbecco's modified Eagle's medium (DMEM) was purchased from *Gibco* Company); PEI (Sigma); Wizard DNA Clean-up System was purchased from Promega Corporation; RNAeasy Kit was bought from *QIAGEN* (Suzhou) Translational Medicine Co. Ltd.; Inverted fluorescence microscope belongs to Leica Microsystems. Conventional biochemical reagents were all analytical reagents made in China.

Biological materials

pEGFP-N1 vector was purchased from Clontech Company; lentivirus vector pmiRZip, modified green fluorescence-free lentivirus vector pmiRZipΔ and HEK293T cells were provided by professor Sun Huaichang of Yangzhou University; Ready-to-use lentivirus packaging plasmids pMD2.G and psPAX2 were purchased from FitGene BioTechnology Co. Ltd.; Duck embryo fibroblast (DEF) was purchased from Beijing ZhongYuan Ltd. (ATCC CCL141); DHAV-1 SH strain was provided by Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences; *DH5α* competent cells and pVL-3CD-P1 plasmids were provided by Jiangsu Key Laboratory for High-Tech Research of Veterinary Bio-pharmacy.

RdRp gene amplification and construction of fusion expression vector

The primer (5' to 3') was designed according to the RdRp gene sequence of DHAV-1 (SH strain, HQ265433). RdRp-F: TTCGAATTCATGGGGAAAGTAGTGAGTAAGC (*Eco*RI); RdRp-R: GGTGGATCCTTGATCATCATGCAAGCTGTGTATG (*Bam*HI). The primer synthesis was carried out by Invitrogen Corporation (Shanghai). The pVL-3CD-P1 plasmid used as template was amplified to acquire the terminator-deleted RdRp gene. Common agarose gel electrophoresis was performed on the PCR product of RdRp gene, and the target gene band was recovered according to the specification of Wizard DNA Clean-up System. Then the recovered product was connected with the RdRp digested by *Eco*RI and *Bam*HI enzymes, as well as pEGFP-N1. Conventional methods were adopted for the transformation and screening of recombinant plasmid. Sequencing was conducted by Invitrogen Corporation (Shanghai).

Detection of RdRp protein expression

HEK293T cells were transfected by pEGFP-RdRp and pEGFP-N1 respectively, with three replicates for each plasmid. After incubation for additional 24 h, cells were examined directly under fluorescence microscope for EGFP expression and total RNA was extracted for RT-PCR amplification of the RdRp-EGFP transcript under the same conditions for RdRp gene amplification.

Sequence design and vector construction

Based on sequencing result of pEGFP-RdRp, a prediction of RdRp-specific siRNAs was carried out utilizing genscript software (http://www.genscript.com). Three were selected from the potential sequences, and shRNA primer was designed according to the requirements of lentivirus vector pmiRZipΔ (Table 1, the matched sequences of siRNA and RdRp were underlined). Primer synthesis was performed by Invitrogen Corporation (Shanghai).

Three pairs of shRNA primer sequences were annealed and cloned into the lentivirus vector pmiRZipΔ digested by *Bam*HI and *Eco*RI enzymes, respectively. These recombinants were sent to Invitrogen Corporation (Shanghai) for sequencing, and the names pRdRp-shRNA1, pRdRp-shRNA2 and pRdRp-shRNA3 were used for corresponding recombinants with correct sequencing result.

Detection of suppression efficiency of plasmid-delivered siRNA

HEK293T cells were cultured in 24-well plate by 10^5 cells per well. After culturing for 24 h, the purified pRdRp-shRNA1, pRdRp-shRNA2 and pRdRp-shRNA3 were separately transfected with pEGFP-RdRp according to the combination modes in Table 2 and the method of literature (Durcher et al., 2002). Each combination had 6 replicates.

Fluorescence microscopy

After transfection for 36 h, the culture plates were observed and

Table 1. Design of sequence with specific interference to RdRp.

Name of shRNA Primer	shRNA sequence (5' to 3')
RdRp-shRNA1–F(18-38)	GATCGCAATATGCAGGTAAGATTCTTCAAGAGAGAATCTTACCTGCATATTGCttttt
RdRp-shRNA1-R	AATTAAAAAGCAATATGCAGGTAAGATTCTCTCTTGAAGAATCTTACCTGCATATTGC
RdRp-shRNA2–F(952-972)	GATCGGGGATGACTGTGTTCTGTCATCAAGAGTGACAGAACACAGTCATCCCCttttt
RdRp-shRNA2 -R	AATTAAAAAGGGGATGACTGTGTTCTGTCACTCTTGATGACAGAACACAGTCATCCCC
RdRp-shRNA3–F(1194-1214)	GATCGGAAGCATTCAAACAGCAACTTCAAGAGAGTTGCTGTTTGAATGCTTCCttttt
RdRp-shRNA3-R	AATTAAAAAGGAAGCATTCAAACAGCAACTCTCTTGAAGTTGCTGTTTGAATGCTTCC

Table 2. Design scheme of transfection.

Group	Vector added	Proportion of vector
1	pRdRp-shRNA1+ pEGFP-RdRp	0.5 μg:0.5 μg
2	pRdRp-shRNA2+ pEGFP-RdRp	0.5 μg:0.5 μg
3	pRdRp-shRNA3+ pEGFP- RdRp	0.5 μg:0.5 μg
4	pmiRZipΔ+ pEGFP-RdRp	0.5 μg:0.5 μg
5	pEGFP-RdRp	1 μg
6	blank	1 μg

photographed under fluorescence microscope to record the fluorescent brightness and quantity of enhanced green fluorescent protein (EGFP) of cells in each well. Then the culture solution was discarded, and the cells were suspended with 100 μL PBS.

Flow cytometry

The cells in each well were collected for all transfection groups, and PBS was added to dilute the cell concentration to 10^9 cells/L. Then 3×10^4 cells in each transfection group were detected with flow cytometer to calculate the average fluorescence intensity of GFP. The extent of inhibition on RdRp protein (n=3) can be calculated by the following formula: Suppression efficiency = (Average fluorescent stregth of GFP in control group- Average fluorescent strength of GFP in experimental group)/ average fluorescent strength of GFP in control group×100%.

Quantitative fluorescence PCR

From each group, the cells in each well were collected. Total RNA was extracted by referring to the specification for Trizol. Reverse transcription was performed for 1 μg of total RNA, followed by quantitative fluorescence PCR, with GAPDH as reference gene. The primers for amplifying RdRp gene in quantitative fluorescence PCR were as follows: RdRp-Qpcr-F: 5'-TTATGGAGCAACTACAGA-3', RdRp-Qpcr-R: 5'-AAGTTACAAGCCTCAATG-3'. Reaction system included 10 μL of Premix Ex Taq TM (2×), 0.4 μL of forward primer and reverse primer (10 μM each), 0.8 μLof 10 μM probe and 1 μL of cDNA, and H_2O was supplemented to reach a 20 μL system. The reaction condition was as follows: 94°C/3 min; 95°C/10 s→55°C/10 s→72°C/15 s, 40 cycles; 72°C/7 min. Dissolution curve was used to analyze the specificity of amplification products. Rotor-Gene3000 Classic (Bio-Rad) was used to collect the Ct values after reaction. Data analysis was performed with $2^{-\Delta\Delta}$Ct method (n=3) (Livak and Schmittgen, 2001).

Preparation of recombinant lentivirus

The shRNA corresponding to siRNA with high suppression efficiency was cloned into the pmiRZip vector using conventional recombinant DNA technology. After sequencing, the qualified recombinant was called pmiRZip-shRNA.

HEK293T cells with good growth status were harvested at 70 to 80% confluence in advance. According to literature (Durcher et al., 2002), 20 μL packaging plasmid MIX and 4 μg siRNA expression vector with strong suppression screened were used for transfection. After 48 and 72 h, supernatant was collected respectively, and centrifuged at 3000 rpm and 4°C for 10 min. Then the supernatant was stored after filtering by 0.45 μm millipore filter. Meanwhile, pmiRZip was also transfected prepare negative recombinant lentivirus.

Purification of recombinant lentivirus

The lentivirus supernatant was fully mixed with 5×PEG8000 concentrating solution at the ratio of 4:1. After placed at 4°C overnight, the mixture was centrifuged at 4000 rpm for 20 min. The supernatant was discarded, and PBS was added to dissolve the lentivirus precipitate. Gradient dilution method (Yin and Liu, 1997) was employed for the titer determination of recombinant lentivirus. After cultured at 37°C and 5% CO_2 for 72 h, the original medium was replaced by 100 μL of fresh one to observe fluorescence expression. The number of fluorescent cells in the last well showing fluorescence expression was counted. The titer of original virus solution = the number of fluorescent cells/virus volume after dilution.

Detection of DHAV-1 $TCID_{50}$ on DEF cells

DEF cells were infected with DHAV-1 SH strains by routine method. 50% tissue culture infection dose ($TCID_{50}$) of the virus was calculated by Karber's method (Yin and Liu, 1997).

Detection of suppression efficiency of recombinant lentivirus - delivered siRNA

Detection of DHAV-1 $TCID_{50}$

DEF cells were inoculated into 24-well plate at the ratio of 1×10^5 cells/well. When the cell density reached about 80%, the cells were inoculated with recombinant lentivirus at a dose of 10 TU/cell, that is, 500 µL for each well. About 48 h later, fluorescence-positive cells appeared. By referring to the dose of 0.001 $TCID_{50}$/TU and method in literature (Zhou et al., 2011), DHAV-1 SH strain was used for infection (n=3). After infection for 24, 48 h, 72, 96 and 120 h, 100 µL of cell supernatant was collected for $TCID_{50}$ determination, respectively. The $TCID_{50}$ of the supernatant of cells infected with DHAV-1 through the transduction of negative recombinant lentivirus was taken as a control, to quantify the suppression of replication of DHAV-1 SH strains by the recombinant lentivirus-mediated siRNA.

Detection of RdRp gene expression

After infection by DHAV-1 SH strains for 120 h, the cells of each well were collected for the extraction of cellular and viral nucleic acid. According to the quantitative fluorescence PCR method above, the expression of RdRp gene was determined with GAPDH as reference gene, so as to judge the suppressing effect of siRNA on the RdRp gene expression in DHAV-1.

RESULTS

RdRp gene amplification and expression

The terminator-deleted RdRp gene was obtained through the PCR with pVL-3CD-P1 plasmid as template. It was proved by the appearance of an expected band of about 1.4×10^3 bp on electrophoresis (Figure 1). The fusion expression vector pEGFP-RdRp was acquired by recombinant DNA technology and was identified through enzyme digestion (Figure 2). The sequencing result indicated that the cloned RdRp gene sequence had 100% homology with the published one of DHAV-1 SH strains in a complete reading frame with GFP gene.

pEGFP-RdRp and pEGFP-N1 were transfected into HEK293T cells, respectively. After 24 h, typical EGFP-positive cells were observed by fluorescence microscopy and an expected 1.4×10^3 bp amplicon was detected by RT-PCR using RdRp-specific primers (Figure 3), suggesting the successful expression of RdRp proteins by the pEGFP-RdRp recombinant.

Construction of lentivirus vector for siRNA expression

Three pairs of shRNA primers were designed according to the requirement of pmiRZipΔ on siRNA expression. By using recombinant DNA technology, the corresponding recombinant plasmids were generated and called pRdRp-shRNA1, pRdRp-shRNA2 and pRdRp-shRNA3 respectively after identification by sequencing.

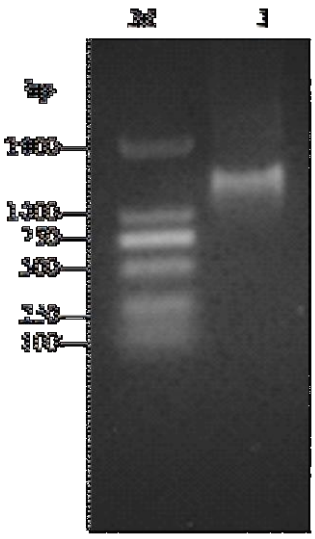

Figure 1. PCR product of DHV-Ⅰ RdRp gene. M: DL2000 DNA Marker; 1: PCR product of RdRp.

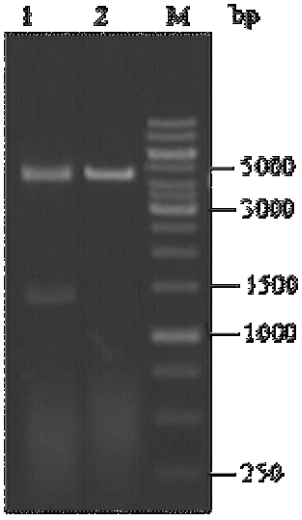

Figure 2. Identification of pEGFP-RdRp by restriction enzyme digestion 1: Double digestion of pEGFP-RdRp by *Eco*RI and *Bam*HI; 2: Double digestion of pEGFP-N1 by *Eco*RI and *Bam*HI M: 1kb DNA Marker.

Detection of suppression efficiency of plasmid-delivered siRNA

The fluorescent brightness and quantity of EGFP of cells in each well were observed under fluorescence microscope 36 h after transfection (Figure 4). The

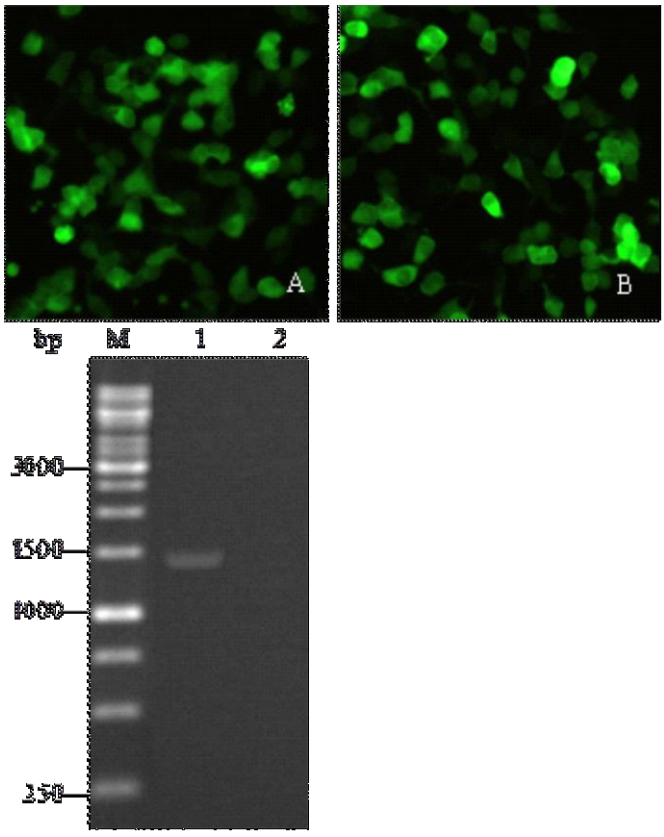

Figure 3. Detection of RdRp-EGFP expression in HEK293T cells (10×10) A: fluorescence of pEGFP-RdRp transfeced cells; B: fluorescence of pEGFP-N1 transfected cells; M: 1kb DNA Marker; 1: RT-PCR amplicon of pEGFP-RdRp transfeced cells; 2: RT-PCR amplicon of pEGFP-N1 transfected cells.

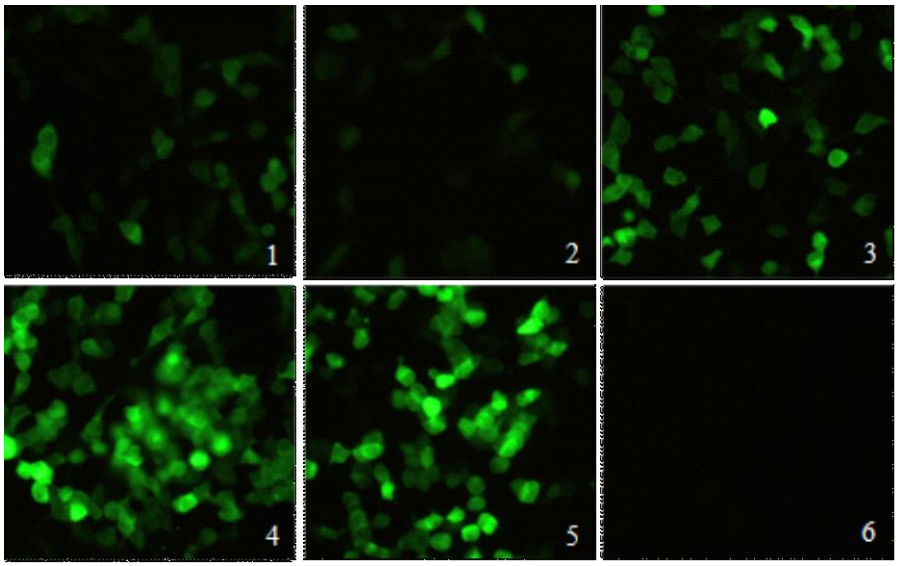

Figure 4. GFP-RdRp expression by fluorescence microscopy (10×10). 1. pRdRp-shRNA1+pEGFP-RdRp transfection group; 2. pRdRp-shRNA2+pEGFP-RdRp transfection group; 3. pRdRp-shRNA3+pEGFP-RdRp transfection group; 4. pmiRZipΔ+pEGFP-RdRp transfection group; 5. pEGFP-RdRp transfection group; 6. Control group.

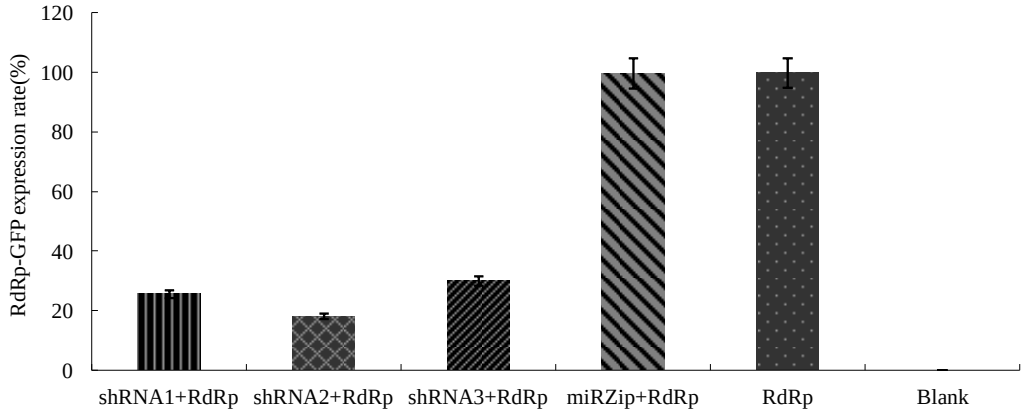

Figure 5. Expression of fusion protein RdRp-GFP by flow cytometry (n=3).

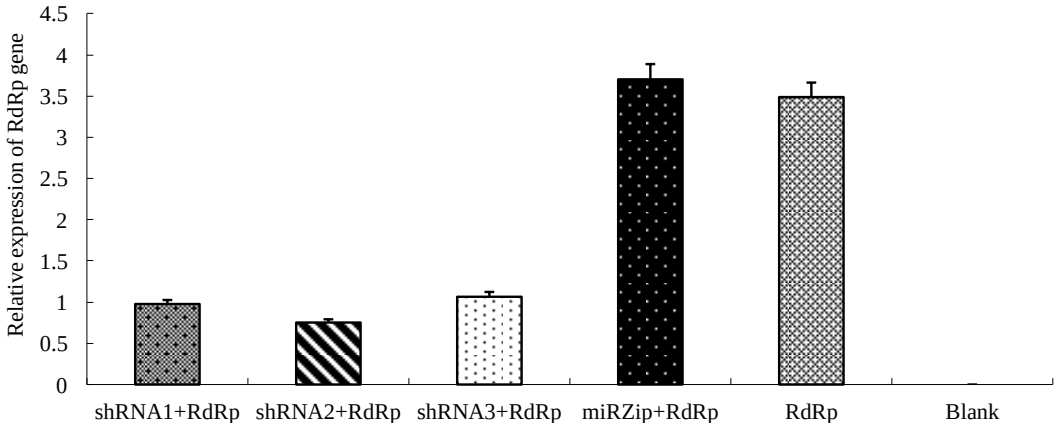

Figure 6. Relative expression of RdRp gene in transfected cells by quantitative fluorescence PCR (n=3).

fluorescent brightness in shRNA transfection attenuated obviously. The expression of fusion protein RdRp-GFP detected by flow cytometry was shown in Figure 5, and it was found that the reduction of protein expression ranged from 69.9 to 81.9%. In the meantime, the cells transfected with different combination of pRdRp-shRNA1, pRdRp-shRNA2, pRdRp-shRNA3 and pEGFP-RdRp were collected for the quantification of RdRp gene. Specific quantitative fluorescent PCR indicated the decrease of RdRp gene expression by approximately 80%, as seen in Figure 6. Among these combinations, pRdRp-shRNA2+pEGFP-RdRp group had the lowest RdRp gene expression, which indicated the suppressing effect of shRNA2 was the strongest.

Preparation of recombinant lentivirus

The recombinant lentivirus corresponding to pmiRZip-shRNA and pmiRZip was packaged in HEK293T cells. After concentrated by PEG8000, the recombinant lentivirus was used to infect HEK293T cells (Figure 7). By gradient dilution method, the titer of original virus solution was determined as 1.3×10^8 TU/mL.

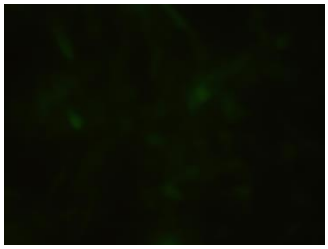

Figure 7. Infection of recombinant lentivirus into HEK293T cells (10×10).

Detection of suppression efficiency of recombinant lentivirus-delivered siRNA

Detection of DHAV-1 $TCID_{50}$

The recombinant lentivirus corresponding to pmiRZip-shRNA and pmiRZip was inoculated into DEF cells at the dose of 10 TU/cell. After 48 h, these DEF cells were

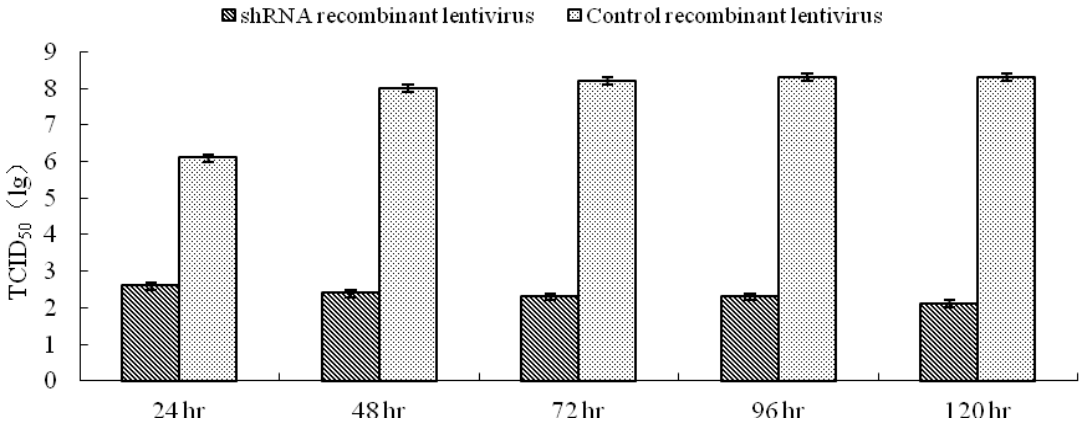

Figure 8. Suppressing effect of shRNA recombinant lentivirus on DHAV-1 determined by $TCID_{50}$ (n=3).

infected with DHAV-1 SH strains, respectively (n=3). After infection for 24, 48, 72, 96 and 120 h, $TCID_{50}$ of the cell supernatant was determined as virus titer. As seen in Figure 8, the suppressing effect on virus had emerged at 24 h, and it could continue to 120 h at least. At 120 h, the $TCID_{50}$ of DHAV-I in the control group was 8.3 lg, while that in the lentivirus infection group with siRNA was only 2.1 lg.

Detection of RdRp gene expression

Total RNA was extracted from the cells after infection by DHAV-1 120 h. The expression of RdRp gene was determined by quantitative fluorescence PCR. Compared with the control group, the RdRp gene expression decreased 89.6% in the lentivirus group carrying siRNA.

DISCUSSION

All known RNA viruses encode a RNA-dependent RNA polymerase, which combines with the protein of hosts (viral protein also needed sometimes) to catalyze RNA polymerization and to modify RNA. Hence, such polymerase is critical to the replication of RNA viruses. Previous studies indicated that the RdRp gene-specific shRNA had stronger viral suppression efficiency than the shRNA specific to the gene of viral structural protein (Gao et al., 2012; Wang et al., 2008). So we selected the RdRp gene of DHAV-1 as target gene to design three shRNAs. Our experimental result showed that under the mediation by plasmid vector, the highest efficiency in silencing RdRp gene expression reached up to 81.9% for the three specific shRNAs. The corresponding recombinant lentivirus reduced the $TCID_{50}$ of DHAV-1 by 6.2 lg and the RdRp gene expression by 89.6%, which agreed well with our expectation. A new idea is found for the clinical control of duck virus hepatitis, which will contribute to the alleviation of huge losses brought by DHAV-1 to breeding industry.

There are several conserved regions (domain or motif) in RdRp protein of various viruses. It is believed that RdRp contains 8 conserved motifs, which can be found in RNA viruses, whether double-strand, positive-strand or negative-strand. These motifs are crucial for RNA replication. The typical GDD motif marks RdRp, and it has been reported that the glycine in GDD motif has important functions (Beerens et al., 2007; Sánchez and Juan, 2005). However, GDD motif is substituted by SDD motif in porcine reproductive and respiratory syndrome virus (PRRSV). When the serine in SDD motif mutated into glycine through artificial induction, PRRSV could be rescued (Zhou et al., 2011). Therefore, we speculate that GDD motif plays an important role in the viral virulence. We designed a specific siRNA containing GDD sequence, that is, shRNA2. The experimental results showed that shRNA2 had higher suppression efficiency on RdRp gene compared with other shRNA, and the corresponding recombinant lentivirus had a strong, long-running suppression on DHAV-I. This implies the potential influence of GDD deletion on viral virulence. Hence, a new control strategy is provided for the control of duck virus hepatitis and other diseases induced by RNA viruses.

In order to achieve the qualitative screening and quantitative judgments of effective shRNA, and exclude the influence from the differences in fluorescence intensity of GFP expressed by various vectors, the lentivirus vectors with or without GFP report gene were both selected for tests. Although an additional procedure of vector modification was introduced, GFP as an indicator could reflect transfection or infection efficiency intuitively, saving a lot of time and labour.

The efficiency of viral gene suppression was generally above 70%. But even at the optimal condition, both gene transfection efficiency of cells and transfection efficiency of recombinant lentivirus could not reach this level. This

suggests that siRNA expressed by infected/transfected cells may have the ability of transcellular transmission (Rausch, 2006). The reason of why the suppression efficiency failed to reach 100% is still unclear, possibly indicating a connection with the RNAi vector expression system, siRNA design software, and cell transfection and infection efficiency. More efforts are needed to clarify the underlying mechanisms.

Conflict of Interests

The authors have not declared any conflict of interests.

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