Effective inhibition of infectious bursal disease virus replication by recombinant avian adeno-associated virus-delivered microRNAs

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INTRODUCTION

Infectious bursal disease virus (IBDV) causes a highly contagious disease in chicks, with heavy losses to the poultry industry worldwide. Control of the disease relies mainly on vaccination, but its protective effect is compromised by the apoptotic effect of live vaccines on the bursa of vaccinated chickens and sequence mutations of IBDV isolates (Snyder, 1990; Sharma et al., 2000). The double-stranded RNA (dsRNA) viral genome consists of two segments, A and B (Murphy et al., 1995). The smaller segment B encodes the 97 kDa VP1 with polymerase and capping enzyme activities, whereas the larger segment A contains a large open reading frame (ORF) encoding a 110 kDa precursor protein that is processed into the mature structural proteins VP2 and VP3 by viral protease VP4 (Boot et al., 2000). Among the viral proteins, VP2 is the major structural protein involved in viral capsid formation, cell entry and induction of protective immune responses (Caston et al., 2001).

RNA interference (RNAi) is a post-transcriptional gene-silencing mechanism in eukaryotes ranging from worms to humans. In this process, the cellular complex Dicer cleaves a dsRNA molecule to yield discrete 21–23 nt small interfering RNAs (siRNAs) or microRNAs (miRNAs), which guide the RNAi-induced silencing complex to cleave the target mRNA (Fire et al., 1998; Meister & Tuschl, 2004). Since its discovery in 1994 as an innate antiviral mechanism, RNAi has been shown to be a powerful strategy against a variety of virus infections (Haasnoot et al., 2003). Previous experiments have shown that plasmid vector-delivered anti-VP1 siRNAs or anti-VP2 miRNAs can efficiently inhibit IBDV replication (Gao et al., 2008; Wang et al., 2009), but in vivo application of the RNAi technology remains to be investigated further.

Adeno-associated virus (AAV) is a small, single-stranded DNA virus that requires helper adenovirus or herpesvirus for productive replication (Atchison et al., 1965; Buller et al., 1981). As a gene transfer vector, AAV has the advantages of non-pathogenicity, broad tissue tropism and long-lasting foreign gene expression, and thus has been used extensively as the gene transfer vector for gene therapy and vaccine development (Durin, 1997). Recent studies have shown that AAV can also act as a delivery vector of siRNAs against human hepatitis B virus (Grimm & Kay, 2007) and coxsackievirus (Fechner et al., 2008). To explore the feasibility of avian AAV (AAAV) as a miRNA-delivery...
vector, in this study we generated recombinant AAVs (rAAAVs) expressing anti-VP1 or anti-VP2 miRNAs and tested their inhibitory effects on IBDV replication.

**METHODS**

**Cells and viruses.** The DF-1 cell line (ATCC CRL-12203) was grown at 37 °C, 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS). IBDV Lukert strain (Lukert et al., 1975) is a tissue-culture-adapted strain, and the wild-type IBDV isolates YEZ and LYG were adapted to DF-1 cells by eight blind passages.

**Construction of miRNA expression vectors.** Construction of the anti-VP2 miRNA expression vector pRFPRNAmiVP2E and the control miRNA expression vector pRFPRNAmiVP2con has been described previously (Wang et al., 2009). The oligonucleotides for short hairpin RNA (shRNA) synthesis are shown in Supplementary Table S1 (available in JGV Online). To select anti-VP1 siRNAs, the VP1 sequence of IBDV Lukert strain was analysed using a web-based siRNA-design tool (http://www.genscript.com), and one candidate siRNA with a sequence identical to a previously described anti-VP1 siRNA was selected for this study (Gao et al., 2008). The miRNA30-like hairpin+ chicken miRNA-flanking sequence was generated by PCR using the siRNA-specific primer pair plus the general primer pair (Supplementary Table S1) according to the instructions for RNAi vector pRFPRNAiC (ARK-Genomics; Fig. 1a). This vector is tailored for miRNA expression in chickens by using a chicken U6 promoter in combination with a miRNA operon expression cassette (Fig. 1b) and using a red fluorescent protein (RFP) cassette as the reporter (Das et al., 2006). After digestion with Nhel and MluI, the PCR product was ligated into the pRFPRNAiC vector digested with the same enzymes and the resultant anti-VP1 miRNA expression vector was designated pRFPRNAmiVP1.

**Generation of rAAAVs.** Single-stranded AAV DNA was extracted from specific-pathogen-free chicken eggs co-inoculated with chicken embryo lethal orphan virus and AAV YZ-1 strain. After denaturation at 95 °C for 10 min and annealing at 65 °C for 6 h, the double-stranded viral genome was recovered from an agarose gel, poly(A)-tailed and inserted into a pCR-2.1 vector, resulting in the recombinant vector pCR-AAAV (Wang et al., 2005; Fig. 1c). The pCR-AAAV vector was digested with PmlI and BsmBI to remove the rep and cap sequences. After separation on an agarose gel, the vector fragment containing the left and right inverted terminal repeats (ITRs) was recovered and blunt-ended with Klenow fragment. The miRNA cassette together with the RFP cassette was excised from pRFPRNAmiVP1, pRFPRNAmiVP2E or pRFPRNAmiVP2con by SalI/BamHI digestion, blunt-ended with Klenow fragment and ligated with the ITR-containing vector fragment, resulting in AAV transfer vector pAITR-RFP, pAITR-RFPmiVP2con, pAITR-RFPmiVP1 or pAITR-miVP2E for the generation of rAAAVs.

![Fig. 1. Schematic structures of the plasmid vectors for generation of rAAAVs.](image-url)
Inhibition of IBDV replication by miRNAs

Characterization of rAAAVs. For morphological analysis of rAAAVs, purified viral particles (50 µl) were observed under a transmission electron microscope after 3% phosphotungstic acid staining. To demonstrate the presence of miRNA cassettes in the viral particles, viral DNA was extracted as described previously (Snyder & Flotte, 2002) and amplified by PCR using a primer pair flanking the miRNA expression sequences (5’-TCCCTGACGCTAGCAGGAG- CCTGAGCAGACACACACAAACATCGAGCCC-3’ and 5’-CC-GATTCTTTAATGCGCGGACCTGACGTCGAGCTGATCGTAAAGAAGCTTACCG-T-3’). PCR was performed in a final volume of 50 µl containing 2 µl viral DNA, 15 pmol each primer and 5 U DNA polymerase (TaKaRa). PCRs were carried out as follows: one cycle of denaturation at 94 °C for 4 min and 30 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. The PCR products (10 µl) were analysed on a 1% agarose gel.

Poly(A)-tailed RT-PCR. Sequence-specific miRNA expression in rAAAV-transduced cells was detected by poly(A)-tailed RT-PCR as described previously (Fu et al., 2005; Ro et al., 2006). Briefly, DF-1 cells were seeded in 35 mm dishes and transduced with different rAAAVs. After incubation for 48 h, small RNAs were isolated using a microRNA purification kit (Norgen Biotech Corp.) and polyadenylated at 37 °C for 60 min in a final volume of 50 µl containing 1 µg RNA, 20 U poly(A) polymerase (New England Biolabs), 5 µl 10 mM ATP and 20 U RNase inhibitor. After acid-phenol/chloroform extraction and ethanol precipitation, reverse transcription was performed in a final volume of 25 µl containing 2 µg RNA, 200 U RevertAid Moloney murine leukemia virus (MMLV) reverse transcriptase (Fermentas Life Sciences) and 1 µg oligo(dT)20 primer flanked by an adaptor sequence [5’-CGAATTCTAGGCTATCCGAGTCGACATGGTGAGCTGACGTCGATCTGACTGTTCC(T)6-3’]. After Tris-phenol/chloroform extraction and ethanol precipitation, miRNA was amplified by PCR using the miVP1-, miVP2E- or miVP2con-specific forward primer (Supplementary Table S1) and a universal primer complementary to the adaptor sequence (5’-CCGAAATTCTAGGCTGAGTCGACGTCGACATGGTGAGCTGACGTCGATCTGACTGTTCC(T)6-3’). Expression of the miRNA in rAAAV-RFP-transduced cells was detected using miVP2E-specific forward primer and a universal reverse primer. PCR was performed as follows: one cycle of denaturation at 95 °C for 10 min and 40 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s, followed by extension at 72 °C for 10 min. PCR products were analysed on a 2% agarose gel.

Flow cytometry. For quantitative analysis of the silencing effects of rAAAV-expressed miRNAs on reporter gene expression, DF-1 cells were seeded in 24-well plates (10 x 103 cells per well) containing a 2% FCS. After overnight cultivation, cells were transduced with different rAAAVs at an m.o.i. of 10 (12 wells for each rAAAV). After incubation for 48 h, each well of wells was transfected with 0.4 µg vector reporter pVP2-GFP as described previously. At 24, 48, 72 and 96 h post-transfection, cells were trypsinized, washed three times with PBS and submitted to flow cytometry analysis (BD Biosciences Clontech) to determine the mean total fluorescence of 3 x 104 cells transduced with each rAAAV.

Semi-quantitative RT-PCR. For quantitative analysis of the silencing effects of rAAAV-expressed miRNAs on IBDV gene expression, DF-1 cells were transduced with each rAAAV as described previously. At 48 h post-transduction, cells were infected at an m.o.i. of 1 with homologous IBDV Lukert strain or heterologous IBDV isolate LYG or YEZ. At 24, 48, 72 and 96 h post-infection, total RNA was extracted and semi-quantitative RT-PCR was performed using a MMLV Reverse Transcriptase kit (Bio Basic) following the manufacturer’s instructions. The PCR primers and program for amplification of VP2 and the internal reference chicken β-actin transcripts have been described previously (Wang et al., 2009). PCR products were separated on a 1.0% agarose gel and the bands of interest were scanned using an ND-1000 Spectrophotometer (NanoDrop Technologies) after ethidium bromide staining.

Virus titration assay. To analyse the inhibitory effects of rAAAV-expressed miRNAs on IBDV replication, DF-1 cells were transduced with different rAAAVs and then infected in triplicate with different IBDV strains as described previously. At 24, 48, 72 and 96 h post-infection, infectious virus in the cell supernatants was titrated on DF-1 cells and the mean 50% tissue culture infection dose (TCID50) was calculated using the method of Reed & Muench (1938).

RT-PCR and sequence analysis. To amplify the miRNA-targeted sequences of the heterologous IBDV isolates, DF-1 cells were infected with isolate LYG or YEZ and total RNA was extracted 48 h after infection using phenol/chloroform. The miRNA targets and flanking sequences were amplified by RT-PCR using an MMLV Reverse Transcriptase kit and the VP1-specific primers (forward primer: 5’-AGGTGCTGTGTCCTCTCGAGCTGATCTGACTGTTCTG-3’; reverse primer: 5’-GGCTGCAGTTCCGTGAGGAGTCC-3’) or VP2-specific primers (forward primer: 5’-CTCCTGTCATGCGAGTCCGAGG-3’; reverse primer: 5’-TTGGACCGTGTTG-3’) as described previously (Wang et al., 2009). Three PCR products for each IBDV isolate were combined and sequenced using the VP1- or VP2-specific forward primer following separation on a 1% agarose gel.

RESULTS

Characterization of rAAAVs

Chloroform-extracted and PEG8000-concentrated viral particles were used to characterize the rAAAVs. By electron

http://virsgm.journals.org
microscopy, rAAAVs showed a typical AAV morphology with a diameter of about 22 nm (Fig. 2a). PCR amplification confirmed the presence of the miRNA expression cassette in rAAAV-RFPmiVP2con, rAAAV-RFPmiVP1 and rAAAV-RFPmiVP2E, but not in rAAAV-RFP (Fig. 2b). Fluorescent microscopy showed typical RFP-positive cells in different rAAAV-transduced DF-1 cells (Fig. 2c) with mean virus titres ranging from 5 × 10^8 to 6 × 10^8 viral particles ml⁻¹. Poly(A)-tailed RT-PCR detected the expected miRNA of 122 bp in rAAAV-RFPmiVP2con-, rAAAV-RFPmiVP1- and rAAAV-RFPmiVP2E-transduced cells, but not in rAAAV-RFP-transduced cells (Fig. 2d).

Silencing effect of rAAAV-delivered miRNAs on reporter gene expression

Reporter assays were used to evaluate the silencing effects of rAAAV-expressed miRNAs on reporter gene expression. Flow cytometry showed a 90.2% inhibition of VP2–EGFP reporter gene expression in rAAAV-RFPmiVP2E-transduced cells, but not in rAAAV-RFP-, rAAAV-RFPmiVP2con- or rAAAV-RFPmiVP1-transduced cells. These gene silencing effects lasted for at least 96 h (Fig. 3).

Inhibitory effect of rAAAV-delivered miRNAs on homologous IBDV replication

Both semi-quantitative RT-PCR and virus titration assays were used to investigate the inhibitory effects of rAAAV-delivered miRNAs on homologous IBDV replication. Semi-quantitative RT-PCR showed that the relative amount of VP2 transcript, which was taken as 100% in rAAAV-RFP-transduced cells, decreased to 14.8% in rAAAV-RFPmiVP2E-transduced cells or 10.4% in rAAAV-RFPmiVP1-transduced cells, but did not decrease in rAAAV-RFPmiVP2con-transduced cells (Fig. 4). Similarly, IBDV titre was decreased by approximately 7.5
log$_{10}$ TCID$_{50}$ in rAAAV-RFPmiVP1- and rAAAV-RFPmiVP2E-transduced cells, whereas a decrease of only 0.3 and 0.5 log$_{10}$ TCID$_{50}$ was detected in rAAAV-RFP- and rAAAV-RFPmiVP2con-transduced cells, respectively (Fig. 5). These inhibitory effects lasted for at least 96 h.

**Inhibitory effect of rAAAV-delivered miRNAs on heterologous IBDV replication**

Virus titration assays were used to investigate the inhibitory effects of rAAAV-delivered miRNAs on heterologous IBDV replication. From 24 h after IBDV infection, the infectious virus titre of the heterologous YEZ isolate decreased by 7.6 or 7.2 log$_{10}$ TCID$_{50}$ in rAAAV-RFPmiVP1- or rAAAV-RFPmiVP2E-transduced cells, respectively, whereas a decrease of only 0.6 TCID$_{50}$ was detected in rAAAV-RFP- and rAAAV-RFPmiVP2con-transduced cells (Fig. 6a). For the heterologous LYG isolate, virus titre decreased by 6.6 or 4.4 log$_{10}$ TCID$_{50}$ in rAAAV-RFPmiVP1- or rAAAV-RFPmiVP2E-transduced cells, respectively, whereas a decrease of only about 0.2 TCID$_{50}$ was detected for rAAAV-RFP- and rAAAV-RFPmiVP2con-transduced cells (Fig. 6b). For both isolates, the inhibitory effects lasted for at least 96 h.

**Sequence analysis of the miRNA-targeted regions of the two heterologous IBDV isolates**

The miVP1 and miVP2E targets and their flanking sequences were amplified by RT-PCR from heterologous IBDV isolate LYG- or YEZ-infected cells using the VP1- or VP2-specific primer pair, and the expected PCR product of 400 or 330 bp was revealed by agarose gel electrophoresis. Sequence analysis of the PCR products showed that the miVP1 and VP2E targets were identical between the homologous Lukert strain and the heterologous isolate LYG or YEZ of IBDV (sequence alignment not shown).

**DISCUSSION**

Previous studies have shown that plasmid vector-delivered anti-VP1 and anti-VP2 miRNAs can effectively inhibit IBDV replication (Gao et al., 2008; Wang et al., 2009). However, the in vivo application of such RNAi technology could be limited due to the large doses required and/or low transfection efficiencies of the plasmid vectors. To explore the feasibility of AAAV as an miRNA-delivery vector in vivo, in this study we generated rAAAVs containing miRNA expression cassettes with relatively high titres, typical morphology and high infectivity in avian cells. Transduction of avian cells with different rAAAVs led to efficient expression of miRNAs in a sequence-specific manner, suggesting the feasibility of using such rAAAVs for further studies of inhibition of IBDV replication.

In the reporter assay, flow cytometry showed that transduction of DF-1 cells with rAAAV-RFPVP2E, but not with rAAAV-RFPVP2con or rAAAV-RFPVP1, led to significant (90.2 %) and long-lasting (>96 h) inhibition of VP2–EGFP expression, indicating the specificity and duration of the gene silencing effect of the viral vector–delivered miRNAs. The inhibitory effects were further investigated using rAAAV transduction and an IBDV infection assay. Both semi-quantitative RT-PCR and virus titration showed that transduction with rAAAV-RFPVP1 or rAAAV-RFPVP2E, but not with rAAAV-RFP or rAAAV-RFPVP2con, led to strong and long-lasting inhibitory effects on gene expression and replication of the homologous Lukert IBDV (Figs 4 and 5). However, the situation was different for the two heterologous IBDV isolates tested. For example, transduction...
with rAAAV-RFPVP1 resulted in slightly lower (compared with the homologous strain) but similar inhibitory effects on replication of the YEZ or LYG isolate, whereas transduction with rAAAV-RFPVP2E produced not only significantly lower but also different inhibitory effects on replication of the two heterologous isolates (Fig. 6). RNA secondary structure among the target and nearby sequences plays an important role in determining the efficiency of RNA interference (Yoshinari et al., 2004). However, our sequence analysis showed that the homologous IBDV Lukert strain shared the same miVP1 and miVP2E targets with the heterologous isolates LYG and YEZ. Therefore, the exact reason(s) for different silencing effects of the miRNAs on different IBDV isolates remains to be determined. One possible reason could be that different amounts of viral transcripts are generated by different IBDV isolates, which has been described in RNAi experiments for other viruses (Ji et al., 2008).

In summary, we have reported the successful generation of rAAAVs for efficient expression of VP1- or VP2-specific miRNA in avian cells. Our results demonstrated that the rAAAV-delivered anti-VP1 miRNA had a strong inhibitory effect on homologous and heterologous IBDV replication, whereas the anti-VP2 miRNA had a strong inhibitory effect on homologous virus replication but different inhibitory effects on heterologous virus replication. Further studies are needed to explore the in vivo utility of this rAAAV-mediated RNAi strategy against IBDV replication.

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**REFERENCES**


