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Small interfering RNAs inhibit infectious bursal disease virus replication in Vero cells

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Summary. – Small interfering RNA (siRNA) molecules are considered to be a promising antiviral therapeutics. This study was performed to analyze the application of siRNA against infectious bursal disease virus (IBDV) replication. Two siRNAs were designed to target common coding sequences of four IBDV proteins. Corresponding vectors were constructed to express anti-IBDV short hairpin RNAs (shRNA) that were tested for their antiviral effect in Vero cells. The results showed that expressed shRNA inhibited the virus replication to a significant extent (92%) as determined by the virus titration in cell culture. This outcome demonstrated the effectiveness of RNA interference (RNAi) based mechanism against the IBDV *in vitro.*

Keywords: infectious bursal disease virus; RNA interference

Introduction

Infectious bursal disease continues to thrive as a significant threat to the commercial poultry industry. It has the ability to remain in the affected farm for a long time and is known for its contagious nature (Van den Berg, 2000). IBDV causes severe immunosuppression *via* destruction of the immature B lymphocytes in bursa of Fabricius (Burkhardt and Muller, 1987). The immunodeficiency caused by the IBDV infection leads to an enhanced susceptibility to the other infections. Moreover, it interferes with the effective vaccination against Newcastle disease, Marek′s disease, and infectious bronchitis (Kibenge *et al.*, 1988). Currently, extensive vaccination program is being followed to control infectious bursal disease, but emerging of the very virulent strains that infect even the vaccinated birds has challenged the vaccination strategy (Chettle *et al.*, 1988; Eterredossi *et al.*, 2004). Outbreaks of this disease by the very virulent strains cause huge economic loss to the poultry industry.

IBDV contains a bisegmented double-stranded RNA and belongs to the genus *Avibirnavirus* of the family *Birnaviridae* (Fauquet *et al.*, 2005). Segment A contains two overlapping ORFs that encode four viral proteins VP2, VP3, VP4, and VP5 (Hudson *et al.*, 1986; Bayliss *et al.*, 1990). Segment B with a single ORF encodes protein VP1 (RNA-dependent RNA polymerase) that is associated with the viral replication (Spies *et al.*, 1987).

The basic mechanism of RNAi is the activation of sequence-specific mRNA degrading machinery. Similarly, RNA duplexes of 21–25 bp long induce a sequence-specific gene silencing in mammals (Elbashir *et al.*, 2001a). The finding concerning a knockdown of some viral genes that can inhibit virus multiplication has generated a great effort to develop siRNA as a possible antiviral agent. This novel approach has been reported to inhibit replication of viruses like foot-and-mouth disease virus, Marek′s disease virus, avian influenza virus, infectious bronchitis virus, porcine reproductive and respiratory syndrome virus, pseudorabies virus, rabies virus, avian leucosis virus, bovine ephemeral fever virus, chicken infectious anemia virus and bovine herpes virus (Chen *et al.,* 2004; Levy *et al.*, 2005; Li *et al.,* 2005; Liu *et al.*, 2005a; Huang *et al.*, 2006; Klupp *et al.*, 2006; Brando *et al.,* 2007; Chen *et al.*, 2007; Chuang *et al.*, 2007; Hinton and Doran, 2008; Narute *et al.*, 2009).

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Abbreviations: IBDV = infectious bursal disease virus; RNAi = RNA interference; siRNA = small interfering RNA; shRNA = short hairpin RNA; CGFP = coral green fluorescent protein

Table 1. shRNA sequences expressed from pRNAT-U6.2 vector

shIBDV325 F	5′-GATCCCGCTACTGCAGGCTAGT GAGTTTCAAGAGAACTCACTAGCCT GCAGTAGTTTTTTCCAAC-3′
shIBDV325 R	5′-TCGAGTTGGAAAAAACTACTGCAG GCTAGTGAGTTCTCTTGAAACT CACTAGCCTGCAGTAGCGG-3′
shIBDV377 F	5′-GATCCCGGTGGCGTTTATGCACTAAT TCAAGAGATTAGTGCATAAACGCCAC CTTTTTTCCAAC-3′
shIBDV377 R	5′-TCGAGTTGGAAAAAAGGTGGCGTT TATGCACTAATCTCTTGAATTAGTGCAT AAACGCCACCGG-3′
shGFP F	5′-GATCCGGCTGACCCTGAAGTTCATCT TCAAGAGA GATGAACTTCAGGGT CAGCTTTTTT GGAAC-3′
shGFP R	5′-TCGAGTTCCAAAAAAGCTGACCCT GAAGTTCATCTCTCTTGAAGATGAACT TCAGGGTCAGCCGG-3′

Nevertheless, the silencing effect is transient in siRNA-transfected animal cells. For the prolonged effect, pol III promoters are used to express the shRNA that is subsequently processed to develop siRNA and degrade target mRNA. The aim of the present study was to determine the inhibitory effect of endogenously expressed siRNA by simultaneous targeting the common coding sequences of four viral proteins of IBDV in cell culture.

Materials and Methods

Virus and cells. African green monkey kidney cells (Vero cells) were maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum and 50 μg/ml gentamicin at 37°C in a humidified atmosphere containing 50 mmol/l of CO_2. Intermediate vaccine strain of IBDV adapted to the Vero cells was used in this study.

Design of siRNA and construction of shRNA expression vectors. The overlapping region of two ORFs of IBDV segment A of Indian isolate KT1/99 (Acc. No. AJ427430) was selected as the potential target region. Eighty sequences of IBDV segment A were retrieved from Genbank (http://www.ncbi.nlm.nih.gov) and aligned by CLUSTAL/W method with *BioEdit* (Hall, 1999). The most conserved sequences between 250 bp to 450 bp were considered as the specific target site. siRNAs were designed with algorithms Deqor scionics (http://clustermpicbg.de/Deqor/deqor.html), GenScript (www.genscript.com), and siDirect (http://design.RNAi.jp). Two siRNA (si325 and si377) sequences were selected based on their specificity after BLAST search against chicken genome (http://blast.ncbi.nlm.nih.gov/Blast). Further, siRNA sequence targeting coral green fluorescent protein (CGFP) described in earlier report (Wise *et al.*, 2007) was utilized in the present study. shRNAs coding template oligonucleotides for the aforesaid siRNAs were designed with a 9 nts loop (TCTCTTGAA) between sense and anti-sense sequences. *BamH*I and *Xho*I restriction sites were included at 5′- and 3′-ends, respectively. Designed oligonucleotides were custom

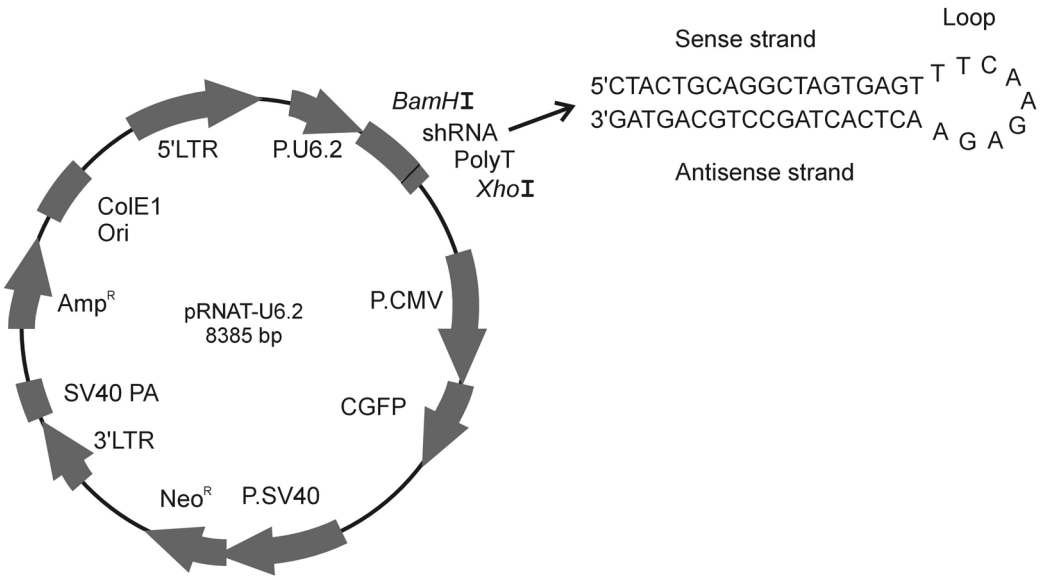

Fig. 1

Scheme of pRNAT-U6.2-shRNA expression vectors

synthesized (IDT, Table 1). Nonspecific shRNA under human U6.2 promoter of the native plasmid was removed by double digestion with *BamH*I and *Xho*I. The annealed anti-IBDV shRNA template oligonucleotides were subsequently cloned into the expression vector pRNAT-U6.2 (GenScript) (Fig. 1). The vector contained pol III promoter, human U6.2 for expression of shRNA construct, and CMV promoter for the co-expression of CGFP. Characterization of the recombinant expression vectors was done by restriction enzyme digestion with *Msp*1 and *Pst*1 followed by the sequencing.

Transfection of cells and viral infectivity titration. Day before transfection, Vero cells were seeded in 24-well plate and grown to 70% confluence. The cells were transfected with two anti-IBDV shRNAs and one nonspecific shCon using Lipofectamine 2000 (Invitrogen). At 6 hrs post transfection, the cells were infected with IBDV at MOI of 0.01 $TCID_{50}$/cell. Next, the cells were incubated in fresh DMEM with 4% FCS. At 48 hrs post infection the culture supernatants were harvested and subjected to three cycles of freezing and thawing. Titration of the infectious supernatants was performed on Vero cells by estimating $TCID_{50}$ according to the method described earlier (Reed and Muench, 1938).

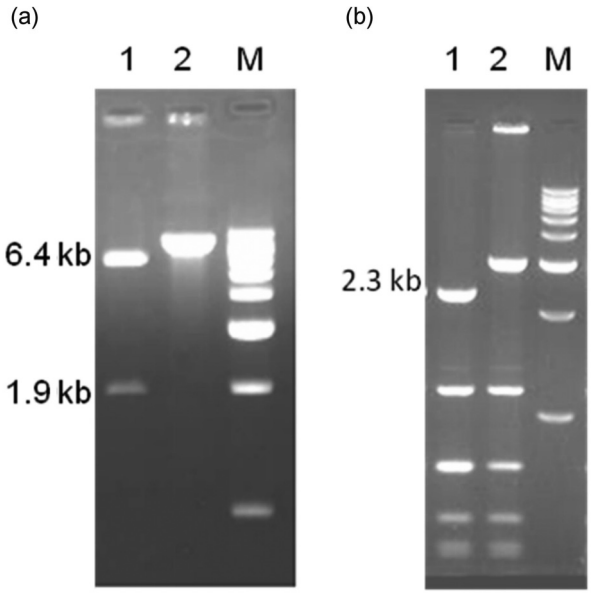

Fig. 2

Restriction analysis of constructed expression vectors

(a) *Pst*I. pRNAT-U6.2-shIBDV 325 (lane 1), pRNAT-U6.2-shRNACon (lane 2), 1 kbp ladder (lane M). (b) *Msp*I. pRNAT-U6.2-shIBDV377 (lane 1), pRNAT-U6.2-shGFP (lane 2), 1 kbp ladder (lane M).

Results and Discussion

Design of siRNA and characterization of expression vectors

To design effective antiIBDV siRNAs, the overlapping region of two ORFs of segment A was chosen as a target. This region contained common coding sequences of four viral proteins VP2, VP3, VP4, and VP5. Hence, it was hypothesized that by targeting of this region a single siRNA would be able to silence the multiple viral proteins and to prove itself as more efficient in the inhibition of virus multiplication. Furthermore, the precise target sequences were chosen by comparison of the conserved region among various IBDV isolates. The selected siRNA showed a minimum of sequence homology with the chicken genome to ensure a pointed antiviral application without side effects on the host. The shRNA forms of two antiIBDV and one antiGFP siRNAs were used to replace a nonspecific shRNA in the expression vector pRNAT-U6.2. The resulting plasmids pRNAT-U6.2-shIBDV325, pRNAT-U6.2-shRNA377, and

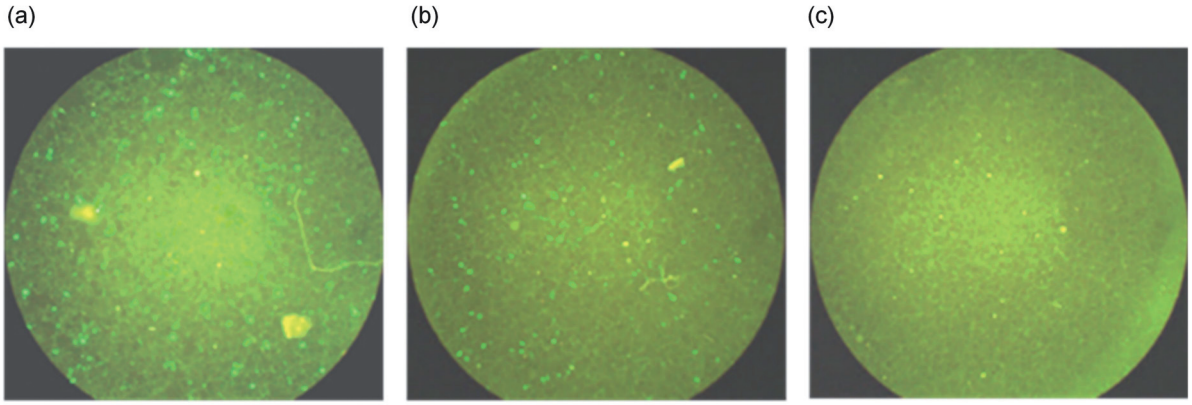

Fig. 3

Inhibition of CGFP expression in Vero cells by a shRNA co-expressed from human U6.2 promoter

Co-expressed shRNAs: shCon (a), shGFP (b), control cells (c).

pRNAT-U6.2-shGFP were analyzed by the restriction digestion with enzymes *Pst*I and *Msp*I. Formation of the desired restriction fragment pattern indicated the presence of shRNA constructs (Fig. 2). Their presence was further confirmed by the sequencing of respective recombinant vectors.

Inhibition of IBDV multiplication by siRNA

On the standardization of transfection it was found that the ratio 1:3 of plasmid DNA to the transfection reagent was optimal. In order to confirm the silencing effect, Vero cells were transfected with the vectors co-expressing shRNA with CGFP gene. Transfected cells were examined under the fluorescent microscope and the presence of expressed shRNA was confirmed. The cells treated with pRNAT-U6.2-shCon showed a higher expression of CGFP as indicated by the higher intensity of fluorescent spots (Fig.3). However, the cells treated with pRNAT-U6.2-shGFP showed fluorescence spots reduced by more than 50% indicating the successful knockdown of CGFP expression. Control cells treated with the transfection reagent alone gave no fluorescent spots (Fig. 3). In the antiviral RNAi experiment, antiIBDV shRNA constructs (pRNAT-U6.2-shIBD325 and pRNAT-U6.2-shIBD377) transfected in Vero cells showed an evident reduction in the virus multiplication compared to the nonspecific shCon construct (pRNAT-U6.2-shCon). Titration of the progeny virus in pRNAT-U6.2-shIBD325-transfected Vero cells revealed a significant reduction in virus titer by 1.07 log unit (91.7%) as compared to the pRNAT-U6.2-shCon-transfected cells. Similarly, in case of pRNAT-U6.2-shIBDV377, the virus titer was reduced by 0.875 $\log_{10,}$ e.g. 86.7% (Fig. 4). This result clearly showed that both siRNAs were able to suppress the IBDV multiplication. However, shIBDV325 was more effective than shIBDV377 in the replication inhibition as demonstrated by the higher reduction of the viral titer. Viral titers in the non-transfected and nonspecific shRNA-transfected cells were consistently comparable in three independent experiments, what indicated that the transfection procedure had no effect on the proliferation ability of IBDV. Recently, in a similar study of DNA vector based RNAi against IBDV, a significant reduction in virus titer by 87.4% was reported by targeting a viral enzyme RNA-dependent RNA polymerase produced by the segment B of IBDV (Gao *et al.*, 2008). In the present study, the siRNA targeting common sequences of four IBDV proteins (VP2, VP3, VP4, and VP5) gave slightly higher reduction in the viral titer (92%). This result indicated that all viral proteins were required in a sufficient concentration to make a large number of functional viral particles. On that account, a simultaneous targeting of multiple viral proteins by a single effective siRNA had higher antiviral effect. Nevertheless, such a deduction requires appropriate comparison of siRNAs from both studies in a single experimental setting.

In conclusion, the present study showed that IBDV was susceptible to the endogenously expressed siRNA-mediated gene silencing. Both siRNAs targeting the common coding sequences of four viral proteins were able to suppress IBDV multiplication. However, further *in vivo* challenge studies are required to determine whether siRNA with appropriate delivery system could control the IBDV infection in susceptible birds.

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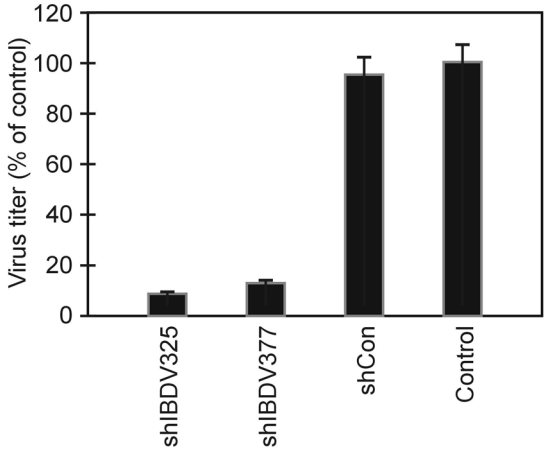

Fig. 4

siRNA mediated inhibition of IBDV replication in Vero cells

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