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Inhibition of avian leukosis virus subgroup J replication by miRNA targeted against *env*

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Abstract No effective vaccine has been developed against the subgroup J avian leukosis virus (ALV-J). The genetic diversity of ALV-J might be related to the *env* gene, therefore, we selected conserved sequences of the *env* gene and designed interference sequence. In this study, microRNAs (miRNAs) were designed and synthesized, corresponding to conserved regions of the *env* gene. These miRNAs were cloned into the linearized eukaryotic expression vector. The recombinant plasmids were transfected into DF-1 cells. After transfection, the cells were inoculated with ALV-J. In reporter assays, the transfection efficiency is 80 % by indirect immunofluorescence (IFA). Expression of the virus envelope glycoprotein was measured by IFA and western blotting assays. The relative expression of *env* gene was determined using quantitative PCR. Our results show that the mi-*env* 231 and mi-*env* 1384 could effectively suppress the replication of ALV-J with an efficiency of 68.7–75.2 %. These data suggest that the miRNAs targeting the *env* can inhibit replication of ALV-J efficiently. This finding provides evidence that miRNAs could be used as a potential tool against ALV infection.

Keywords ALV-J · *env* · RNA interference · miRNA

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Introduction

Avian leukosis (AL) is a general term for a variety of neoplastic diseases in poultry caused by a C-type avian retrovirus. AL virus (ALV) has been classified into 10 subgroups, designated A–J. ALV belongs to RNA virus that causes immune suppression and tissue tumors in infected fowls. ALV-J was first isolated from Dorking fowl in the early 1990s [1].

RNA interference (RNAi) is an effective tool for silencing targeted genes. It involves endogenous or exogenous double-stranded RNA (dsRNA)-mediated degradation of specific mRNA sequences. In the process, the cellular complex Dicer cleaves a dsRNA molecule to generate discrete 21–23 nt small interfering RNAs (siRNAs) or microRNAs (miRNAs), which guide the RNAi-induced silencing complex (RISC) to cleave the target mRNAs [2, 3]. In the past, siRNAs have been successfully studied for the inhibition of viral replication. Infection of cells with human immunodeficiency virus (HIV) may be hindered by inhibiting the expression of CD4 and CD8a, both HIV receptors. Inhibiting expression of their co-receptors, CXCR4 or CCR5, or Gag, the virus structural protein, can result in reduced levels of HIV infection [4]. In some studies, transfection of siRNAs designed against hepatitis C virus (HCV) inhibited expression of virus-specific proteins, and protected cultured cells against HCV RNA [5, 6]. In another study, hepatitis B virus (HBV) replication was successfully inhibited following expression of HBV siRNAs transfected into the mouse liver [7]. Hu et al. adopted siRNAs designed against the ALV *gag* gene and demonstrated a reduced capacity for virus replication [8]. More recently, endogenous miRNA genes have been found to more effectively result in RNAi effects [9, 10]. Artificial miRNA has been developed [11, 12]. It has been

demonstrated that expression of miRNA vectors is more effective and less toxic than regular siRNA vectors [13, 14]. MiRNAs are a class of endogenous non-coding single-stranded RNA molecules, 19–24 nucleotides (nt) in length, that have been highly conserved during evolution. Through specific complementary base pairing with target gene mRNA, miRNAs cause the degradation of target mRNAs, or inhibit their translation. This negatively regulates the expression of target genes [15, 16]. Mo et al. [17] indicated that ALV-B replication was significantly inhibited after knockdown of ALV-B *tvb* and *env* genes.

ALV, especially ALV-J, brings about enormous economic losses in the poultry industry. The virus has spread rapidly worldwide, with the ability to be transmitted vertically and horizontally. However, chicks are immunologically tolerant to ALV. To date, no effective vaccine has been developed against ALV. Especially, genetic and antigenic variations with sequence changes in the variable regions of the *env* gene of ALV-J have been observed [18–20]. In order to explore a new approach to inhibit ALV-J, here we report on vector-delivered miRNA molecules that are studied for their inhibitory effects on ALV-J replication at a cellular level. This study provides not only an experimental basis for the development of a new anti-ALV-J strategy but also for a new approach to study ALV-J infection and replication.

Materials and methods

Viruses and cells

The SD strain of ALV-J was isolated from poultry in Shandong (China) and stored at the Harbin Veterinary Research Institute (Harbin, China). The DF-1 cell line was provided by Zhigao Bu at the Harbin Veterinary Research Institute (Harbin, China).

Reagents

The eukaryotic expression vector pcDNA6.2-GW/EmGFP-miR, *Escherichia coli* Top10 cells, and Lipofectamine 2000 transfection reagent were purchased from Invitrogen (Carlsbad, CA, USA). Ligase and reverse transcriptase were purchased from TaKaRa (Dalian, China). The fluorescence quantitative PCR (qPCR) kit was purchased from TaKaRa Technology Co., Ltd. (Dalian, China). Plasmid and viral RNA extraction kits were purchased from Shanghai Watson Biotech Co. (Shanghai, China). FITC-conjugated goat anti-mouse IgG and horseradish peroxidase (HrP)-labeled goat anti-mouse IgG were purchased from Zhongshan Goldbridge Biotechnology Co. (Beijing, China). The anti-ALV-J monoclonal antibody (clone JE-9) [21] was kindly provided by Professor Qin Aijian (Yangzhou University, China). All other chemicals were of analytical reagent grade.

miRNA design and the eukaryotic expression vector

The reference sequences of the *env* gene (AY234051; AY234052; AY897218; AY897220; AY897221; AY897222; AY897223; AY897224; AY897225; AY897226; AY897227; AY897228; AY897229; AY897230; AY897231) were obtained from the National Center for Biotechnology Information (NCBI) and compared with Clustal W Method of the DNAStar software. Four pairs of miRNAs sequences (Table 1) were designed based on the conserved regions of the *env* gene using software available at http://www.rnaidesigner.invitrogen.com/rnai. MiRNA sequences were synthesized by Shanghai Health Bioengineering (China), denatured and then annealed into double strands. Double-stranded DNA (dsDNA) was then inserted into a linearized expression vector, pcDNA6.2-GW/EmGFP-miR, to construct recombinant plasmids containing the target miRNAs. These four RNAi expression vectors were designated mi-env231, mi-env1278, mi-env1294, and mi-env1384. All recombinant plasmids have been sequenced to confirm the sequences inserted.

Transfection and preparation of ALV

DF-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 100 U/ml of penicillin and streptomycin, and 5 % (v/v) fetal calf serum (PAA GOLD, Austria). Cells were grown to 70 % confluence before sub-culturing, and then used to seed cell culture plates at a density of $3–5 \times 10^5$ cells/cm^2. When sub-cultured cells had grown to 80 % confluence, they were transfected with the appropriate RNAi expression plasmid using Lipofectamine 2000^{TM} kit. 6 h later, culture media was aspirated and replenished with DMEM containing serum and 5 % peracetic acid (PAA). At 48 h after transfection, transfection efficiency was determined by the expression of green fluorescent protein (GFP) observed under a fluorescence microscope. Transfected cells were inoculated with ALV-J 100 TCID50. DF-1 cells were harvested 72 h after viral infection, and the inhibitory effect was determined using an indirect immunofluorescence assay (IFA), western blotting, and qPCR. The negative control, null vector control, and test groups were randomized for analysis.

Measurement of cell apoptosis by flow cytometry

At 48 h after transfection, DF-1 were digested and prepared by washing twice with PBS, and 5 μl Annexin V-rhodamine isothiocyanate (RITC) and PI were added to the cells, combined, and incubated for 15 min at room temperature. The rate of apoptosis was monitored by FACSAria (Becton–Dickinson).

Table 1 Oligonucleotide sequences of pre-miRNAs

	Sequence (5′ to 3′)
mi-*env* 231	TGCTGTGAAGGAACATACAGAGAAGACTTTTGGCCACTGACTGACTCTTCTCTATGTTCCTTCA
	CCTGTGAAGGAACATAGAGAAGAGTCAGTCAGTGGCAAAACTCTTCTCTGTATGTTCCTTCAC
mi-*env* 1278	TGCTGTAAAGGCTCTAAATACAACCCGTTTTGGCCACTGACTGACGGGTTGTATAGAGCCTTTA
	CCTGTAAAGGCTCTATACAACCCGTCAGTCAGTGGCCAAAACGGGTTGTATTTAGAGCCTTTAC
mi-*env* 1294	TGCTGCAAACAGCATGCTTGATAAAGGTTTTGGCCACTGACTGACCTTTATCACATGCTGTTTG
	CCTGCAAACAGCATGTGATAAAGGTCAGTCAGTGGCCAAAACCTTTATCAAGCATGCTGTTTGC
mi-*env* 1384	TGCTGTTGATAGGCATTCCACAGTATGTTTTGGCCACTGACTGACATACTGTGATGCCTATCAA
	CCTGTTGATAGGCATCACAGTATGTCAGTCAGTGGCCAAAACATACTGTGGAATGCCTATCAAC

Table 2 Primers used to amplify target genes

Target gene	Primer sequence	Product size (bp)	Annealing Temperature (°C)
Env	Forward ACCTCACCATTCCGCACCT	241	52.3
	Reverse CTTTATAGCACACCGAACC		
β-actin	Forward TCCCTGTATGCCTCTGGTC	250	55.0
	Reverse TCTCTCTCGGCTGTGGTGG		

Determination of ALV-J virus titer

Strain SD was inoculated into DF-1 cells. Cultures were subjected to three cycles of freeze-thawing, and then centrifuged (7,000×*g*, 4 °C, 5 min). Supernatants were collected and serially diluted tenfold to 10^{-8}, then inoculated into 96-well plates containing DF-1 cells. Four parallel wells were used for each dilution. 5 days later cells were fixed in methanol (−20 °C, 30 min), then washed three times (10 min per wash) with phosphate-buffered saline (PBS) containing 0.1 % (v/v) Tween 20 (PBST). The JE-9 monoclonal antibody (100 μL) at a 1:200 dilution was added to each well. Cells were incubated at 37 °C for 1 h and washed with PBST three times (10 min per wash). Goat anti-mouse IgG conjugated to FITC was added (1:200 dilution), and cells incubated at 37 °C for 45 min in the dark. Cells were washed five times with PBST. The number of wells emitting fluorescent light was counted using a fluorescence microscope, and the $TCID_{50}$ was calculated using the Reed–Muench method [22]. The exact dilution was calculated using the following formula: [62.5 % (percentage of cells with a $TCID_{50}$ value above 50 %) − 50 %] / [62.5 % (higher than 50 % of the percentage) – (less than 50 % of the percentage)] = 0.2.

Expression of the envelope glycoprotein by IFA

Virus strain SD was used to inoculate DF-1 cells transfected with recombinant plasmid using the methods described above. 5 days later, cells were treated with the JE-9 monoclonal antibody and rhodamine-labeled goat anti-mouse IgG/TRITC (Fluorescent secondary antibody). Selected fields of view were photographed under a fluorescence microscope.

Western blot analysis

Approximately 72 h after transfection with the miRNA recombinant plasmid, DF-1 cells were collected. Total protein was extracted using a total protein extraction kit (BestBio, China) and quantified using a UV spectrophotometer. Sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) electrophoresis (10 %) was performed. We used a sample volume of 300 μg/well. Samples were transferred to nitrocellulose membranes, blocked with 5 % skim milk in TBST buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05 % Tween-20) at 4 °C overnight, and then washed three times with PBST. The JE-9 monoclonal antibody was added and cells were incubated at 37 °C for 1 h. Mouse anti-chicken GAPDH monoclonal antibody was added and incubated at room temperature for 1 h, followed by three washes with PBST. HrP-labeled anti-mouse antibody was added and cells were incubated at 37 °C for 45 min. After washing with PBST five times, cells were stained with 3,3′-diaminobenzidine (DAB) solution [6 mg of DAB, 10 mL of Tris-buffered saline (TBS), 0.1 mL of 3 % H_2O_2] and then scanned using an Alphalmager HP.

Determination of the mRNA expression levels of gene fragments by quantitative PCR

Using the ALV *env* sequences published in GenBank, primers were designed against the conserved regions of *env*

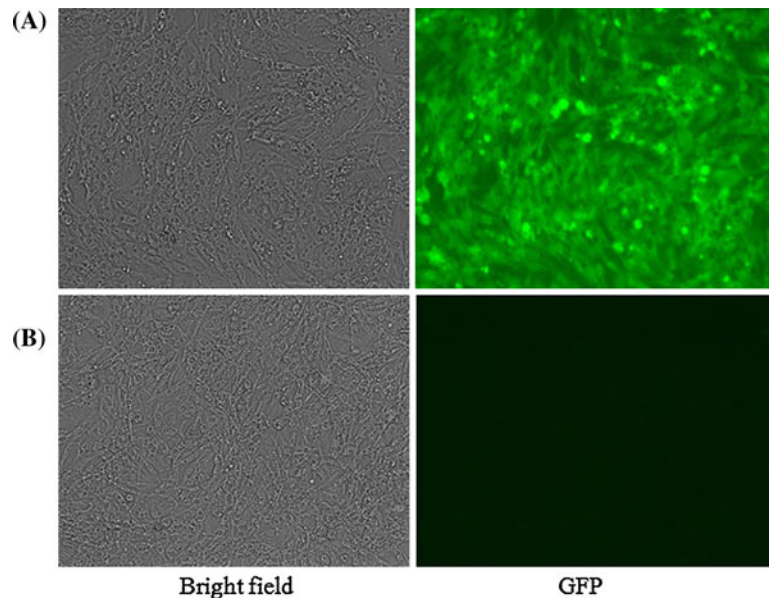

Fig. 1 IFA analysis of the transfection efficiency after 48 h. **a** Cells with the Lipofectamine 2000; **b** Cells transfected the reporter plasmid pcDNA6.2-EmGFP

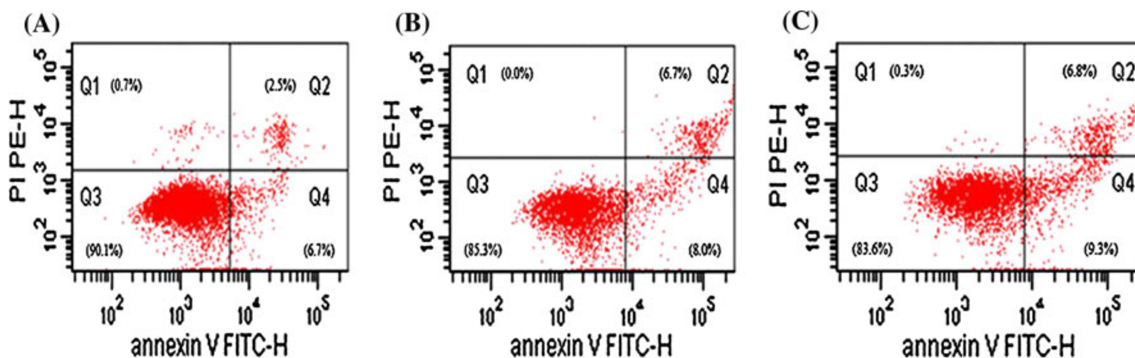

Fig. 2 Representative Annexin-V/PI flow cytometry data for after transfection 48 h. **a** Cells of the control group. **b** Cells with the pcDNA6.2-EmGFP. **c** Cells with the pcDNA6.2-EmGFP-miR. Quadrants Q1, Q2, Q3, and Q4 report the ratio of necrotic cells, late-stage apoptotic cells, normal cells, and early-stage apoptotic cells, respectively

Table 3 Results of the $TCID_{50}$ calculations

Cells	Cell observation		Total cell number of holes		Fluorescence holes disappear	
Percentage	Degree of dilution	Fluorescence holes appear	Fluorescence holes disappear	Fluorescence holes appear	Fluorescence holes disappear	Total number of cells holes
10^{-3}	8	0	21	0	21	100 %
10^{-4}	8	0	13	0	13	100 %
10^{-5}	5	3	5	3	8	62.5 %
10^{-6}	0	8	0	11	11	0
10^{-7}	0	8	0	19	19	0
10^{-8}	0	8	0	27	27	0

gene using Oligo 6 primer design software. The *env* gene and the β-actin gene were amplified by PCR using SD strain cDNA as template (Table 2).

Total RNA was extracted from cells 72 h after infection of ALV-J into DF-1 cells. The total RNA was reverse transcribed to cDNA with ExScript™ RT Reagent Kit (Perfect Real Time) (TaKaRa). The qPCR assays were performed for *env* genes using the SYBR GREEN kit (SYBR® Premix Ex Taq™, TaKaRa, Dalian, China) and primers listed in Table 2. Fluorescence signals were

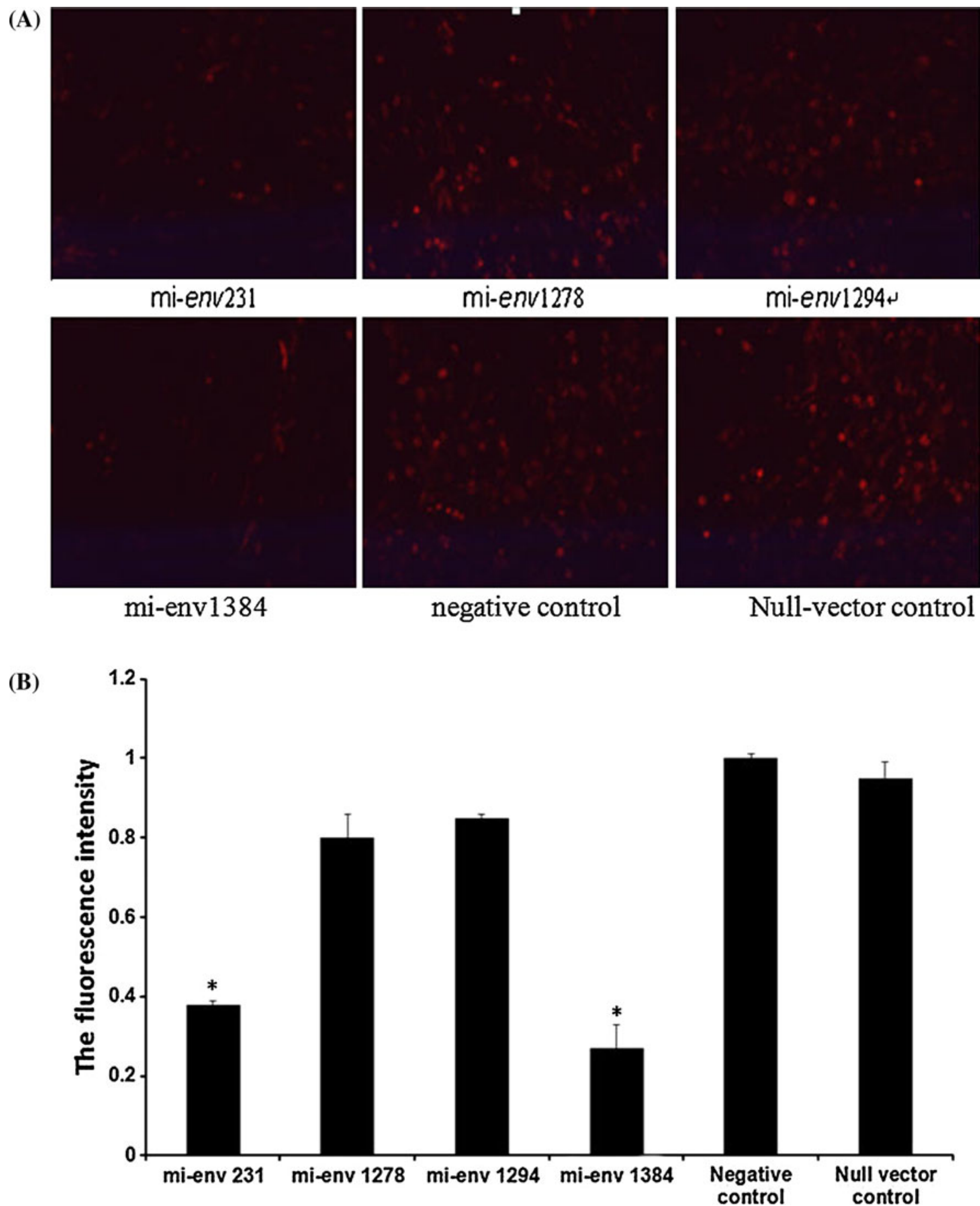

Fig. 3 Inhibition of recombination plasmids to ALV-J by IFA detection. **a** Cells transfected with the recombinant plasmid pENVs and their inhibitory effects against ALV-J, as determined by the IFA. **b** The fluorescence intensity of cells transfected with the recombinant plasmid PENVs and their inhibitory effects against ALV-J, as determined by the IFA. Data are presented as mean ± S.E.M. of three independent experiments, each performed in triplicate. Statistically significant differences compared with negative controls ($P < 0.05$)

recorded at the end of each extension step. Q-PCR data were analyzed using the comparative CT method (ΔΔCT) [23]. β-actin from DF-1 cells was chosen as a reference gene for internal control. Differences between the CT values of the target gene(*env*) and the internal control ($\Delta CT = CT_{target} - CT_{internal\ control}$) were calculated to normalize the differences in the amount of total cDNA added to each reaction and the efficiency of the Q-PCR. The negative control was used as a reference for each comparison. Differences between the ΔCT of each *env*

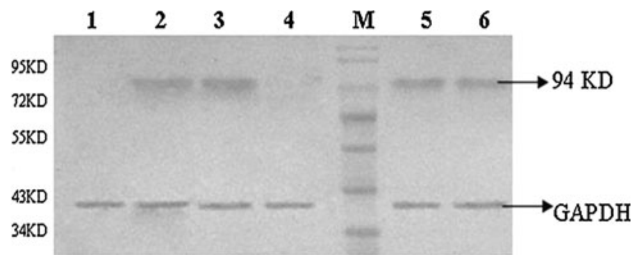

Fig. 4 ALV-J envelope glycoprotein expression of DF-1 cells in each in of the groups. Western blot of infected culture treated with miRNA. *Lane M* protein ladder; *lane 1* mi-*env* 231; *lane 2* mi-*env* 1278; *lane 3* mi-*env* 1294; *lane 4* mi-*env* 1384; *lane 5* negative control; *lane 6* null vector control

expression plasmid and reference sample ($\Delta\Delta CT = (CT_{target} - CT_{internal\ control})env$ plasmid-$(CT_{target} - CT_{internal\ control})$NC) were calculated. The expression level of the target gene could be calculated by $2^{-\Delta\Delta CT}$ and the value stood for an n-fold difference relative to the negative sample.

Statistical analysis

Results are presented as the mean ± standard deviation (s). The *t* test was performed using SPSS 13.0 statistical software. A *P* value less than 0.05 was considered statistically significant.

Results

Identification of the transfection efficiency IFA

The plasmids used for transfection were purified using the QIAGEN plasmid Midi Kit (Qiagen, Germany). After transfection 48 h, typical fluorescence-positive cells were observed by fluorescence microscopy. As Fig. 1 shows ~80 % of the transfected cells are positive cells, indicating transfection efficiencies under the optimized conditions.

Effect of transfection on apoptosis of DF-1

To determine whether miRNAs transfection leads to cytotoxicity in DF-1 cells, flow cytometry analysis was performed to test the effect of transfection on the apoptosis of DF-1. Annexin-V/PI staining revealed that no significant differences were observed in the proportion of normal cells in three groups (83.6 % compared with 90.1 and 85.3 %) (Fig. 2.). The result showed that miRNAs transfection did not significantly increase the apoptosis of DF-1.

IFA and determination of virus titer

IFA results were observed after 5 days using a fluorescence microscope. The numbers of wells with or without fluorescence were recorded (Table 3). Virus $TCID_{50}$ values were between 10^{-5} and 10^{-6}. Therefore, the toxic potential of the virus was $10^{5.2}$ $TCID_{50}$/0.1 mL, corresponding to $10^{6.2}$ $TCID_{50}$/mL.

IFA detection of ALV envelope protein

As shown in Fig. 3a, b, IFA revealed that the recombinant plasmids, mi-env231 and mi-env1384 significantly reduced fluorescence intensity ($P < 0.05$). The use of tandem plasmids indicated significant inhibition of the expression of the ALV-J envelope glycoprotein in cells (60.4–70.5 %). However, no significant differences were detected among

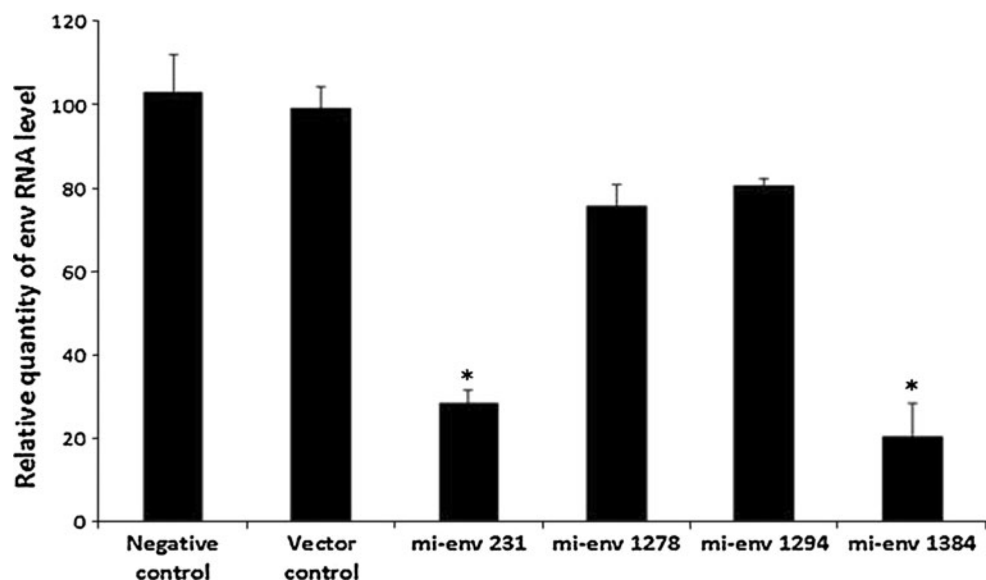

Fig. 5 Quantitative analysis of the silencing effects of the miRNAs on ALV-J replication. Total RNAs were extracted at 72 h after infection for q-PCR analysis of viral gene expression using ALV-J-specific primers. β-actin gene served as the internal reference. The data shown represent the mean value for three separate experiments; standard deviation indicated by *error bars*. Statistically significant differences from the negative control are indicated by *($P < 0.05$)

other groups compared with the negative control and null vector groups.

Western blot analysis of the ALV-J envelope glycoprotein

Using western blot analysis (Fig. 4), mi-env231 and mi-env1384 resulted in significantly decreased levels of ALV-J envelope glycoprotein expression ($P < 0.05$). There were no significant differences between the remaining groups and the negative control or null vector groups.

qPCR determination of ALV-J mRNA levels

Melt-curve analysis confirmed specific amplification of real-time PCR products. Using cDNA templates, the efficiency of the PCR reactions for β-actin and *env* were shown to be similar, permitting the relative abundance of the integrated mRNA to be estimated. The qPCR analysis for *env* gene expression (Fig. 5) showed that in cells transfected with plasmids mi-env231 and mi-env1384, expression levels differed significantly from that observed in the negative control and null vector control groups ($P < 0.05$). The expression of ALV env was inhibited 68.7 and 75.2 % by mi-env231 and mi-env1384 transfection 72 h after infection, respectively, compared with the levels of viral RNA in the negative control and null vector control groups.

Discussion

Ever since its discovery in the United Kingdom in the late 1980s [24], ALV-J has become highly prevalent in chickens and has caused serious problems for the poultry industry worldwide [25]. In China, ALV-J has become a major problem in layer chickens since 2008 [26]. ALV-Js have evolved rapidly in pathogenicity, which results in a change in host range and tumor spectrum. The genetic diversity of ALV-J might not be restricted to the *env* gene, genetic rearrangements, and high mutation rates were also found in noncoding genomic regions [27, 28].

RNAi has since been accepted by the scientific community as a potential clinical tool against infectious diseases [29, 30]. These infectious diseases include: HIV type 1 [31–33]; HCV [34]; HBV [35]; severe acute respiratory syndrome coronavirus [36]; and influenza A virus [37]. RNAi strategies have been applied to the inhibition of ALV replication by a number of research groups. Mo et al. [17] successfully inhibited ALV-B replication induced by a retroviral vector by targeting the miRNA of the ALV-B *env* gene and its receptor encoded by the *tvb* gene. Meng et al. [38], successfully inhibited ALV-J replication by targeting the miRNA of ALV-J *gag* genes. ALV is an RNA virus with a genome of approximately 7.6 kb. The proviral genome of ALV-J contains three major genes: *gag*; *pol*; and *env*. These three genes encode the major structural protein of the virus, RNA-dependent DNA polymerase, and the envelope glycoprotein, respectively. Genetic and antigenic variations, with sequence changes, in the variable regions of the *env* gene of ALV-J have been observed [39]. These alterations increase the difficulty of diagnosis and prevention of the disease. In this study, the ALV-J *env* gene was used as a target gene and RNAi was used to suppress its expression.

We constructed miRNA expression vectors targeting the *env* gene of ALV-J. The results demonstrated that mi-env231 and mi-env1384 could significantly reduce the expression of target gene mRNA, and expression of the envelope glycoprotein at a cellular level, with the highest inhibition rate of 75.2 % observed for mi-env1384. The miRNAs targeted against the *env* genes could successfully inhibit ALV-J replication. The identification of anti-ALV genes could potentially lead to the development of an ALV vaccine.

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