

# shRNA-triggered RNAi inhibits expression of NDV NP gene in chicken embryo fibroblast

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**Abstract** RNA interference (RNAi) technology is a powerful tool for identifying gene functions. Chicken embryo fibroblast (CEF) is an ideal model for studying the interaction between avian viruses and their hosts. To establish a methodological platform for RNAi studies in CEF, three plasmid vectors expressing short hairpin RNAs (shRNAs) targeted against the Newcastle disease virus (NDV) NP gene were constructed. One of them, *ndv1*, was proven effective on blocking viral replication in CEF and chicken embryos. Four hours prior to infection with NDV, the CEF was transfected with the plasmids by Silent-fect. An unrelated shRNA sequence (HK) was used in mock transfection. The expression of a potent shRNA resulted in up to 2.3, 21.1 and 9.8 fold decreases in NP gene expression at 3, 6 and 9 h post infection in CEF, respectively. The *ndv1* was able to completely inhibit the replication of the virus in CEF within 48 post infection. Furthermore, the pathological changes in CEF caused by NDV were delayed, and the degree of pathological changes was lighter compared with the mock transfection in the presence of *ndv1*. When the complex of shRNA-Silent-fect and NDV was co-injected into the allantoic cavity of 10-day-old embryonated eggs with 10<sup>5</sup> or 10<sup>6</sup> ELD<sub>50</sub> NDV, NDV replication was decreased by 94.14% and 62.15% after 17 h, respectively. These findings suggest that the newly synthesized NP protein is critical for NDV transcription and replication and provide a basis for identifying the functions of viral genes and screening for effective siRNAs against viruses in CEF and chicken embryo by RNAi.

**Keywords** RNAi, short hairpin RNA, Newcastle disease virus, chicken embryo fibroblast, embryonated chicken egg

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## 1 Introduction

RNA interference (RNAi) is a process by which double-stranded RNA (dsRNA) directs sequence-specific degradation of mRNA in eucaryotic cytoplasm. In this process, the antisense (guide) strand of short double-stranded RNAs (small interfering RNA, siRNA) is incorporated into an RNA-induced silencing complex that can either suppress protein expression or direct degradation of messenger RNAs that contain homologous sequence(s) (Hannon, 2002; Pickford and Cogoni, 2003). It has been reported that 21 bp siRNAs synthesized artificially could trigger an effective RNAi process in mammalian cells without inducing interferon response (Elbashir et al., 2001). However, the 19 bp small hairpin RNAs (shRNAs) with a loop structure, when inserted into intracellular expression vectors, could be recognized and cut into siRNA by RNA endonucleases, triggering the RNAi process (Brummelkamp et al., 2002). Replication of viruses in cells can induce RNAi, which, in turn, suppresses viral replication (Vance and Vaucheret, 2001; Li, 2002). The intermediate product of RNA virus replication, dsRNA, could be cut into siRNAs by intracellular RNA endonuclease and form a complex (RISC), resulting in corresponding gene silencing (Hannon, 2002). A large number of studies have demonstrated that RNAi can effectively inhibit the proliferation of various human and animal viruses *in vivo* and *in vitro*, including human immunodeficiency virus type 1 (HIV-1), hepatitis B virus (HBV), hepatitis C virus (HCV), SARS-associated coronavirus, swine fever virus (SFV), respiratory syncytial virus (RSV), influenza A virus (IAV), vesicular stomatitis virus (VSV), dengue fever virus (DFV), parainfluenza virus (PIV), poliovirus and avian infectious bronchitis virus (IBV) (Barik, 2004; Saleh et al., 2004; Haasnoot et al., 2003; Caplen, 2003; Liu, 2005). Many avian viruses not only cause great economic loss to poultry products,

but are also potential threats to public health. The Newcastle disease virus (NDV) is a nonsegmented, negative-stranded RNA virus, belonging to the mumps virus genera of the paramyxoviridae family. This virus can infect many kinds of poultry and cause lethal diseases. Currently, ND is still one of the most important diseases of poultry in China. Chicken-embryo fibroblast (CEF) is a primary cell commonly used as a host for the replication of many viruses. To our knowledge, there have been no reports about RNAi in CEF. We constructed plasmid vectors that express shRNAs targeted against the NDV NP gene, and performed RNAi experiments in CEF cells and chicken embryos. The aims of our study were to provide useful information on gene functions of avian viruses, mechanisms of virus-host interaction, and control of viral infections.

## 2 Materials and methods

### 2.1 Design of shRNA and construction of expression plasmids

Three highly reserved regions were selected as the candidate targets by analyzing 15 full-length mRNA sequences of the NDV NP gene available in the GenBank. Those target sites are located at 183–202, 191–210 and 233–252 bp, respectively. To design DNA templates expressing single shRNA, a 19 bp sense strand was connected to the anti-sense strand by using 9 non-complementary nucleotide acids, forming a stem-loop. The DNA template was then inserted into the pGenesil-3 plasmid that carries a U6 promoter and the red-fluorescent protein (RFP) gene. The recombinant plasmids were named as ndv1, ndv2 and ndv3, respectively. The G+C contents of the shRNAs were 52.64%, 52.64% and 42.11%, respectively. There was no mismatch with the other regions of the NP gene sequence of the chosen NDV strain. Also, the DNA sequence had no homology with any known avian or human genomes. A plasmid vector HK, which contained an unrelated sequence (5'-GACTTCATAA-GGCGCATGC-3'), was constructed as a negative control and sequenced by Genesil Biotechnology Co., Ltd (Wuhan, China).

### 2.2 Chicken embryo fibroblasts culture and viral infection

CEFs were prepared from 10-day-old SPF chicken embryos (Nanjing Pharmaceuticals and Medical Instrument Factory, Nanjing, China) in 250 mL flasks as previously described (Yin and Liu, 1997). For siRNA introduction, the logarithmic-phase CEF cells were trypsinized, washed, and grown in a 48-well culture plate at  $5 \times 10^4$  cells per well at 37°C in a 5% CO<sub>2</sub> atmosphere until an 80% coverage rate of cells was achieved. The

shRNA was mixed with liposome transfection reagent (Silent-fect, BioRad Corporation) gently at a ratio of 1 : 3 (μg : μL) and incubated at room temperature for 30 min. The mixture was added into the CEF cell culture with 1 μg shRNA and 200 μL antibiotic-free and serum-free DMEM medium (Gibco/BRL Corporation, USA) per well and incubated at 37°C in 5% CO<sub>2</sub> atmosphere for 1.5 h. The culture medium was then replaced with DMEM containing 3% FBS and cultured for 4 h. Following that, the culture medium was removed and  $1 \times 10^4$  ELD<sub>50</sub>/200 μL NDV (F48E8, China National Supervision Institute of Veterinary Medicine) was added to each well. After absorption for 1 h at 37°C, the inoculums were removed and DMEM supplemented with 3% FBS was added into each well and the cells were cultured at 37°C with 5% CO<sub>2</sub>. The same treatment was applied to the negative control, plasmid HK. Each treatment was performed in triplicates.

### 2.3 NDV and shRNA inoculation in chicken embryos

For each inoculation, 3 μg shRNA was mixed with 9 μL Silent-fect as described above. The mixture was combined with either 10<sup>5</sup> or 10<sup>6</sup> ELD<sub>50</sub> F48E8 virus and immediately injected into the allantoic cavity of 10-day-old embryonated chicken eggs. The eggs were incubated at 37°C for 17 h and the allantoic fluid was harvested to measure virus titer. The same treatment was applied to the negative control, plasmid HK. Each experiment was performed in triplicates.

### 2.4 RNA extraction, reverse transcription, and real-time PCR

CEF cells were transfected with ndv1, ndv2, ndv3 or HK, respectively and were infected with the F48E9 virus. At 3, 6 and 9 h after infection, the culture supernate was removed and the cells were lysed by using the Trizol reagent (TaKaRa). The RNAs were isolated and cDNAs synthesized by using an RT kit (TaKaRa) following the manufacturer's protocol. The cDNAs were then used for real-time PCR by using NP gene-specific primers (forward: 5'-CCCAACGAGTGTCTGAGGAG-3', reverse: 5'-CTA-CCGCTCTCATAAGGTCCAA-3'). Real-time RT-PCR was then performed with SYBR<sup>®</sup>Premix Ex Taq<sup>™</sup> (TaKaRa, Japan) in a 25 μL reaction mixture with 1 μmol/L final primer concentration. The reaction was then performed for 40 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 40 s. Chicken β-actin gene specific primers (5'-CTGTGCCCATCTATGAAGGCTA-3', 5'-ATTTCTCTCTCG GCTGTGGTG-3') were used for real-time PCR to detect chicken β-actin mRNA. The reaction was then performed for 40 cycles at 95°C for 3 min, 94°C for 30 s, 62°C for 30 s, and 72°C for 40 s. The quantities of PCR products were examined using the ABI7300 real-time PCR system (Applied Biosystems). The relative changes in

mRNA expression of the NP gene were calculated using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001).

## 2.5 Virus titer assay

At different time points after infection, the supernatants were harvested from the infected cultures and the virus titer was determined in V-bottomed 96-well plates. The following equation was used to calculate the inhibition ratio, which represents the inhibitory effect of the shRNA on NDV proliferation:

$$\text{Inhibition ratio (\%)} = \frac{\text{HA mean value of positive group}}{\text{HA mean value of negative group}} \times 100\%.$$

## 2.6 Observation of cytopathogenic effect

Observations of the cytopathogenic effect (CPE) in CEF cells were made at different time points after infection.

## 3 Results

### 3.1 CPE induced by the F48E8 virus was postponed and alleviated by ndv1

The virus-induced CPE could be observed at 16 h post infection with the F48E9 virus, including expansion of intercellular space, presence of particle-like structures in cytoplasm, cytomorphosis and presence of syncytium. With the progress of the infection process, disintegration and exfoliation of the cells and plaques could be observed. Most of the cells exfoliated at 48 h post-infection. Compared with the negative control, the occurrence of CPE was postponed and

the morphologic changes were alleviated by introducing ndv1 after infection with the F48E9 virus. On the other hand, ndv 2 and ndv 3 showed no effect on CPE. The differences in CPE and morphology of the groups are shown in Table 1 and Figure 1.

**Table 1** Effects of shRNAs on CPE induced by F48E8 virus in CEF cells

groups	CPE caused by NDV						
	time post-infection with NDV/h						
	16	20	24	28	32	36	48
ndv1	+	++	++	++	++	+++	+++
ndv2	+++	++	+++	+++	++++	++++	++++
ndv3	+++	+++	+++	++++	++++	++++	++++
HK	+++	+++	+++	+++	++++	++++	++++

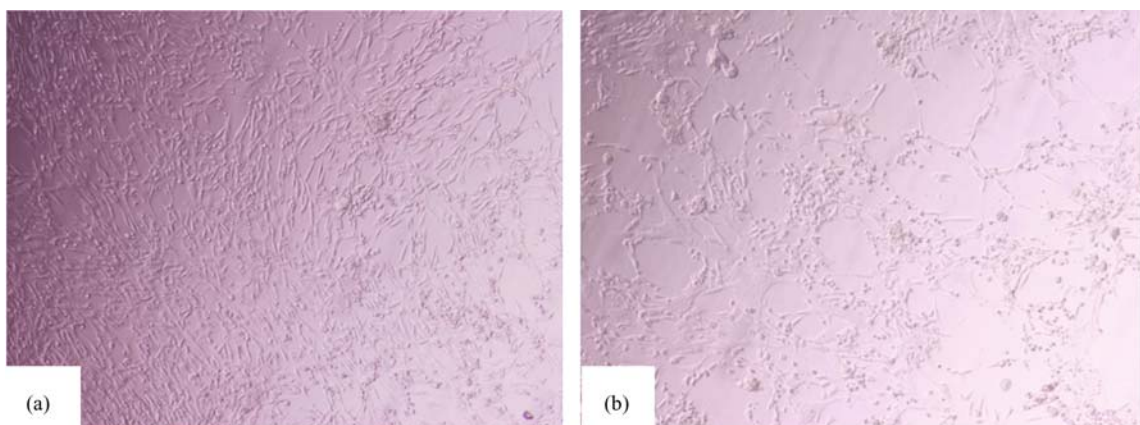
Note: +: less than 25% of the cells showed CPE; ++: 50% of the cells showed CPE; +++: 75% of the cells showed CPE; ++++: more than 90% of the cells showed CPE.

### 3.2 Inhibitory effect on NDV NP gene expression in CEF by ndv1

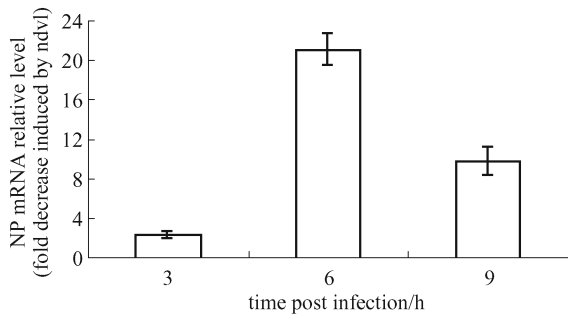
Compared with the negative control, the NDV NP gene expression levels were lowered by ndv1 by 2.3-fold, 21.1-fold and 9.8-fold at 3, 6 and 9 h post infection, respectively (Fig. 2). The NP gene expression levels in CEF cells introduced with ndv2 and ndv3 were not detected.

### 3.3 Inhibitory effect on proliferation of NDV in CEF cells by ndv1

Virus titers were determined by HA assay and the results indicate that the virus titer of the negative control increased gradually with time since infection, while the virus titers could not be detected in the cell culture



**Fig. 1** Syncytium formation and exfoliation of the CEF cells induced by NDV were prevented by the ndv1(100×). (a) CEF cells introduced with the ndv1 24 h post infection; (b) CEF cells administered with the HK after infection.



**Fig. 2** NP-specific shRNA inhibited the accumulation of specific mRNA. CEF cells were transfected with ndv1 and infected with 10<sup>4</sup> ELD<sub>50</sub> NDV F48E8. mRNAs were isolated at 3, 6, and 9 h after infection and mRNA expression levels of the NP gene were evaluated using real-time PCR. The NP mRNA levels at each time point were normalized to the level of β-actin mRNA in the same sample. The relative levels of NP mRNA are shown as mean ± SD (*n* = 3, SD at 3, 6 and 9 h were 0.40, 1.63 and 1.42, respectively).

suspension inoculated with ndv1 until 48 h post infection. It suggests that ndv1 was able to inhibit the proliferation of NDV in CEF and the inhibitory rate was 100% (Table 2).

**Table 2** Inhibitory effect of ndv1 on NDV proliferation in CEF

groups	virus titer/2 <sup>n</sup>						
	time post infection/h						
	16	20	24	28	32	36	48
ndv1	0	0	0	0	0	0	0
HK	0	2 ± 1.15	2 ± 0	2 ± 0	4 ± 1.15	4 ± 0	8 ± 0

**3.4 Inhibitory effect on NDV proliferation in chicken embryo by the ndv1**

The HA titers of the virus in allantoic fluid of different groups post infection are shown in Table 3.

**Table 3** HA titers of the virus in allantoic fluid

dosage of the virus/ELD <sub>50</sub>	virus titer/2 <sup>n</sup>					
	ndv1			HK		
10 <sup>5</sup>	4	4	2	32	16	128
10 <sup>6</sup>	16	16	64	128	64	64

As shown in Table 3, the virus titer was decreased significantly after administration of ndv1, which suggests that ndv1 was able to block the replication of NDV in chicken embryos. Compared with the negative control group (HK), 3 μg of ndv1 resulted in a virus decrease by 94.4% in allantoic fluid when infected with 10<sup>5</sup> ELD<sub>50</sub> F48E9 viruses and by 62.5% when infected with 10<sup>6</sup> ELD<sub>50</sub> F48E8 viruses.

**4 Discussion**

The interference effect of siRNAs could be affected by several factors such as the GC content, the secondary structure of the target sequence and properties of the terminal nucleotides of siRNAs. Ui-Tei *et al* (2004) introduced several principles to design efficient siRNA sequences: (1) A/U at the 5' end of the antisense strand; (2) G/C at the 5' end of the sense strand; (3) at least five A/U residues in the 5' terminal one-third of the antisense strand; and (4) the absence of any GC stretch of more than 9 nt in length. In the current study, we analyzed 15 full-length mRNA sequences of the NDV NP gene available in the GenBank and divided these sequences into two clusters, FJ-1-85 (partial cds. ACCESSION, AF512535, VERSION, AF512535.1, GI:21314540 CDS <1...373) and JS-5-01 (partial cds. ACCESSION, AF512534 VERSION AF512534.1 GI:21314538, CDS <1...373). The FJ-1-85 and JS-5-01 have highly homologous sequences between 1–373 bp. Three shRNAs targeted at the NDV NP gene were designed based on the sequence in these regions. The results indicate that shRNA targeting at the region between 183–201 bp of the NP gene could effectively inhibit the expression of the target gene, while the other two shRNAs had no obvious interference effect. Further studies are needed to screen for effective siRNAs.

RNAi is an ancient intracellular defense mechanism protecting organisms from attack by external genes. Successful introduction of siRNA into cells is the prerequisite for its interference effect. Cationic liposome is the most commonly used transfection reagent when performing RNAi experiments. However, although the efficiency of transfection for passage cells usually reaches 100%, the liposome reagent shows a much poorer efficiency and stronger toxicity to the primary cells (Agrawal *et al.*, 2003). CEF cell is the most commonly used primary cell for culture of many avian viruses and some human viruses. In previous studies, we compared the cytotoxicities of three kinds of cationic liposomes to four types of cells and found that the cationic liposomes showed a lower cytotoxicity and broader safe concentration range to passage cells such as MDCK, but a higher cytotoxicity to CEF. Moreover, the degrees of cytotoxicity to CEF cells of the three cationic liposomes were obviously different. Silent-fect, which had shown the lowest cytotoxicity (Li *et al.*, 2005), was used in the present study. In addition, we found that the tolerance of CEF to transfection reagents was enhanced when subcultured 2–3 times. This finding helped us complete the RNAi experiments using NDV in CEF successfully.

RNAi is one of the most important strategies for gene function analysis. By using RNAi, Bitko and Barik (2001) proved that the P gene of RSV, VSV and HPIV-3 is one of the most important parts of viral RNA-dependent RNA polymerase, based on the finding that inhibition of the P

gene resulted in a dramatic reduction of almost all viral RNAs. It was also proven that the F gene, not the SH gene, of RSV is closely related to syncytium formation (Heminway et al., 1994; Heminway et al., 1994). Furthermore, by using RNAi, Barik (2004) confirmed that a syncytium forms only when both the F and HN proteins, encoded by the F gene and HN gene of the HPIV-3, are present in the infected cells, and apoptosis of the VSV infected cells is associated with the M protein of the virus. NP proteins encoded by the mumps virus of the paramyxovirus family play an important role in the replication of the virus and therefore have attracted much attention (Horikami et al., 1992; Myers et al., 1999). The replication of the Sendai virus depends on the interaction between the NP and P proteins (Horikami et al., 1992). Curran et al. (1993) further confirmed this mechanism and found that the terminal amino acid of the P protein is the molecular chaperone of the NP protein, which prevents non-specific assembly of the NP protein. This interaction is believed to be the basis of transcription switching. It is generally thought that the NDV genome is the template both for replication and transcription, which are two separate stages in the viral replication cycle. The initial NP protein (NP<sup>0</sup>) acts as a switch of replication and transcription, generates packaging signals in the process of new-synthesized RNA packaging and determines polymerases to continue replication of the genome or start transcription (Curran et al., 1995). In the present study, when the NP gene expression was suppressed, the synthesis of NP protein was blocked in the CEF cells and chicken embryos and the virus titer decreased significantly (even becoming undetectable). It indicates that NDV replication was suppressed. The results demonstrate that the NP gene plays a crucial role in the replication of NDV.

Based on plasmids or viruses, several types of vector systems stably expressing shRNA in mammalian cells have been constructed to exhibit long-term RNAi effect. These vector systems make RNAi technology easier to perform. Plasmid vectors are commonly used because they are easy to construct (Lamb and Kolakofsky, 1996). In the present study, NDV proliferation in CEF cells and chicken embryos was inhibited by the plasmid vector encoding shRNA. This proves that RNAi mechanism is present in the CEF cells as it is in mammalian cells. CEF cells and chicken embryos are commonly used hosts for propagation of a variety of animal viruses and several human viruses. Therefore they are suitable for study of gene functions of both the viruses and chickens. As the complete sequences of the chicken genome as well as the virus have been available recently, RNAi may have great application value in the future.

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