Original Paper

Intervirology

Intervirology 2007;50:63–70
DOI: 10.1159/000097391

Received: November 1, 2005
Accepted after revision: February 14, 2006
Published online: November 30, 2006

# Inhibition of SARS-CoV Gene Expression by Adenovirus-Delivered Small Hairpin RNA

Xue Zhang Kailang Wu Xin Yue Ying Zhu Jianguo Wu

State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan, PR China

**Key Words**
Small hairpin RNA · SARS-CoV · Adenovirus · Nucleocapsid protein · Envelope protein

**Abstract**
***Objective:*** Severe acute respiratory syndrome (SARS) is a highly contagious and lethal disease caused by a new type of coronavirus, SARS-associated coronavirus (SARS-CoV). Currently, there is no efficient treatment and prevention for this disease. We constructed recombinant adenoviral vectors that can express shRNAs, which inhibited the expression of SARS-CoV genes effectively in mammalian cells. ***Methods:*** In this study, we designed several plasmids that express small hairpin RNA molecules (shRNA) specifically targeting to the genes encoding for the SARS-CoV nucleocapsid (N) protein and envelope (E) protein, respectively. Effective shRNA molecules to the viral genes were screened and identified, and then constructed into adenovirus vectors. The effects of adenovirus-delivered small hairpin RNA on SARS-CoV gene expression were determined by RT-PCR, Western blot, and luciferase activity assays. ***Results:*** The levels of viral mRNAs and viral proteins of the targets were significantly decreased or completely inhibited in cell lines after being infected with the recombinant adenoviruses that expressed specific shRNA molecules. ***Conclusions:*** Since many cell types can be efficiently infected by adenovirus, recombinant adenoviruses could serve as an alternative powerful tool for shRNA delivery and for gene suppression, especially when the targeted cells are resistant to transfection by DNA or RNA. With availability of high titers of adenoviruses and uniform and rapid infection, this approach would have foreseeable wide applications both in experimental biology and molecular medicine.


## Introduction

Between the end of 2002 and June 2003, the severe epidemic disease called severe acute respiratory syndrome (SARS) broke out in China and more than 30 other countries. It was known that SARS coronavirus (SARS-CoV) is a novel human coronavirus and responsible for SARS infection [1–4]. SARS-CoV is an enveloped positive-stranded RNA virus, and its genome is composed of about 29,700 nucleotides [5, 6]. The SARS genome encodes 23 putative proteins that are typical of coronavirus such as 5′-replicase (rep), spike (S), membrane (M), envelope (E), and nucleocapsid (N)-3′ [7]. The N protein of SARS-CoV is 422 amino acids long, sharing only 20–30% homology with the N proteins of other coronaviruses [5, 6]. Previous studies indicated that the N proteins of other coronaviruses have a variety of functional activities [8], including participation in transcription of the viral genome, the formation of viral core, and packaging viral

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Ying Zhu / Jianguo Wu
State Key Laboratory of Virology, College of Life Sciences, Wuhan University
Wuhan 430072 (PR China)
Tel. +86 27 6875 4979, Fax +86 27 6875 4592
E-Mail yingzhu@whu.edu.cn or wu9988@vip.sin.com

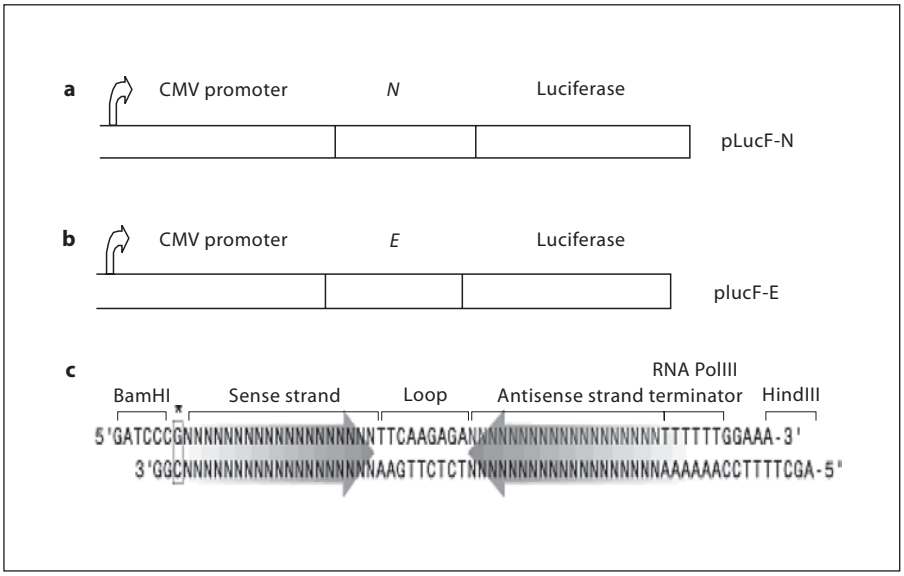


**Fig. 1.** Schematic diagrams of reporter plasmids. The two reporter plasmids were constructed, in which the target sequences of N (pLucF-N) (**a**) or E (pLucF-E) (**b**) was in frame with the luciferase report gene and the expression of the fusion genes were under the control of the CMV promoter. (**c**) Primary sequence of shRNA is shown.

RNA [9, 10]. However, no similar functional roles of SARS-CoV have been reported except that SARS-CoV N may selectively activate AP-1 pathway [11]. Furthermore, SARS-CoV N protein is capable of self-association through a C-terminal 209-amino-acid interaction domain [12, 13]. The E protein is another important structural protein of SARS-CoV. More recently, it was shown to play a major role in coronavirus assembly [14, 15].

RNA interference (RNAi) is an ancient evolutionarily conserved process [16–19]. Ever since synthetic 21–23 nucleotide short interfering RNA (siRNA) was shown to induce efficient RNAi in mammalian cells, siRNA has been routinely used in gene silencing by transfection of chemically synthesized siRNA. Specific inhibition of cellular mRNA by RNAi can be triggered in mammalian cells by the introduction of siRNA. Currently, RNAi technology is reported as an ideal tool to inhibit infectious virus replication in host cells, including poliovirus [20], HIV-1 [21, 22], flock house virus, Rous sarcoma virus [23], dengue virus [24], hepatitis C virus [25] replicons, influenza virus [26], hepatitis B virus [27, 28] and SARS-CoV [29, 30].

Adenovirus vectors have been widely used for the expression of trans-genes under both clinical and experimental conditions [31]. Adenovirus has several unique features, such as ability to infect both dividing and non-dividing cells, lack of cell-mediated immune response against the vector, ability to integrate into a host chromosome or persist episomally. In this report, we describe the construction of a recombinant adenoviral vector that can introduce expression of shRNAs intracellularly and inhibit the expression of SARS-CoV genes effectively.

## Materials and Methods

*Plasmid Construction*

The following two pairs of primers were used to amplify N and E gene from the full-length cDNA of SARS-CoV (SARS-CoV WHU, GenBank accession No. AY394850), respectively.

N gene:

P01: 5′-AGCTGGATCCATGTCTGATAATGGACCCCAATCAAAC-3′ (sense)

P02: 5′-AGCTGAATTCCATCATGAGTGTTTATGCCTGAGT-3′ (antisense)

E gene:

P03: 5′-AGCTGGATCCATGTACTCATTCGTTTCGGAAGAAAC-3′ (sense)

P04: 5′-AGCTGAATTCTTAGTTCGTTTAGACCAGAAGATC-3′ (antisense)

The PCR products were cloned into BamHI and EcoRI sites of pCMV-tag2B (Stratagene) to generate pCMV-N and pCMV-E.

We also used the following two pairs of primers to amplify the fragments carrying part of N or part of E gene:

N fragment:

P05: 5′-CAATTAGATCTATGTCTGATAATGGACCCCAATC-3′ (sense)

P06: 5′-GTTATGTCGACTGCCTGAGTTGAATCAGCAGAA-3′ (antisense)

E fragment:

P07: 5′-CTTCACAGATCTATGTACTCATTCGGTCCG-3′ (sense)

P08: 5′-CAGAGTGTCGACGTTCGTTTAGACCAGAAGATC-3′ (antisense)

The PCR products were cloned into *Sal*I and *Bgl*II sites of plucF [32] to generate plasmid plucF-N (fig. 1a) and plucF-E (fig. 1b), in which the N or E gene was fused in frame with the luciferase gene and the expression of the fusion gene was driven by the CMV promoter. All constructs were confirmed through DNA sequencing.

*Generation of shRNA Expression Vectors*

Target sequences for shRNA were selected according to the web-based criteria and further analyzed by BLAST research to avoid significant homology with endogenous cellular genes.

To construct single shRNA expression vector, two 64-nt primers, each containing a 19-nt target sequence in the sense and antisense strand from different regions of the N gene or E gene (fig. 1c), were synthesized from Invitrogen. Four regions of N and four regions of E gene were selected respectively as the target sites of shRNA in this study. The sequences and locations are listed as following:

5′-GGAGGAACTTAGATTCCCT-3′
(N1shRNA, 28303–28321)
5′- GCTAACAAAGAAGGCATCG-3′
(N2shRNA, 28493–28512)
5′-GAAATTCAACTCCTGGCAG-3′
(N3shRNA, 28704–28723)
5′-GAACAAACCCAAGGAAATT-3′
(N4shRNA, 28958–28977)
5′-GTAAAACCAACGGTTTACG-3′
(E1shRNA, 26270–26289)
5′-GCGTGTTAAAAATCTGAAC-3′
(E2shRNA, 26296–26315)
5′-GAACTCTTCTGAAGGAGTT-3′
(E3shRNA, 26311–26330)
5′-GATCTTCTGGTCTAAACGA-3′
(E4shRNA, 26333–26352)

For each shRNA, sense and antisense oligonucleotides of self-complementary hairpin sequences, which contain cohesive ends for *Bam*HI and *Hin*dIII at the 5′- and 3′-ends, were synthesized and annealed by heating at 95°C for 10 min and slowly cooled down to room temperature. Then, eight annealed oligonucleotides were cloned into the *Bam*HI and *Hin*dIII sites of pSilencer-2.1-U6 plasmid (Amibion) according to the manufacturer's instructions (pN1shRNA, pN2shRNA, pN3shRNA, pN4shRNA, pE1shRNA, pE2shRNA, pE3shRNA, pE4shRNA). The sequences of the constructs were confirmed by DNA sequencing.

*Generation of Recombinant Adenoviruses*

To enhance the delivery efficiency of shRNAs, recombinant adenoviruses were generated as following steps: pN3shRNA, pE4shRNA and pCtrl plasmid were digested with *Eco*RI and *Hin*dIII to release small fragments containing U6 promoter and targeting sequences, which were then ligated into pAdTrack at *Eco*RI and *Hin*dIII sites to generate recombinant plasmid ADN3, ADE4 and ADCtrl, respectively. The recombinant pAdTracks were linearized with *Pme*I and transformed into BJ5183 cells which carry the pAdEasy-1 plasmid. Positive clones were selected and confirmed by PacI digestion. Plasmids from correct clones were amplified by transforming into DH5α cells. The resulting adenoviral DNAs were linearized with *Pac*I and transfected into 293T cells in a six-well plate using Sofast$^{TM}$ transfection reagent (Xiamen Sunma Biotechnology Co. Ltd, PR China) to produce recombinant adenoviruses ADN3, ADE4 and ADCtrl, respectively. Eight days after transfection, the recombinant virus was amplified and titered according to the manufacturer's instructions.

*Cell Lines and Transfections*

293T and COS7 cells were maintained in Dulbecco's modified Eagle medium (Gibco/BRL) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum at 37°C under 5% $CO_2$. Cells were seeded onto plates at a density of $1.0 \times 10^5$ per 24-well plate or 6-well plate and grown to confluence reaching approximately 60% at the time of transfection. Cells were then transfected with 0.1 or 0.4 μg of plasmid which contains N or E gene together with 0.45 or 1.2 μg shRNA expression plasmids, using Sofast$^{TM}$ transfection reagent. The cells were harvested 48 h after transfection. For adenovirus infection, cells were incubated with recombinant virus at MOI of 10–20 at 37°C. After adsorption for 1–2 h, fresh growth medium was added and cells were placed in the incubator for an additional 2–3 days.

*Luciferase Assay*

293T and COS7 cells were co-transfected with reporter plasmids and shRNA expression plasmids. Cells were washed with PBS and lysed with luciferase cell culture lysis reagent (Promega). 10 μl of the cell lysates and 100 μl of luciferase assay substrate (Promega) were mixed and fluorescence intensity was detected by the luminometer (Turner, TD20/20). Assays were performed in triplicate, and expressed as means ± SD relative to vector control as 100%.

*RNA Isolation and RT-PCR Assay*

293T and COS7 cells were co-transfected with pCMV-N or pCMV-E plasmid and shRNA expression plasmids. The total RNA was then extracted from transfected cells by Trizol Reagent (Invitrogen) according to the method described in the manufacturer's manual. Reverse transcriptions were performed with total RNA as the template. Specific mRNA was amplified by RT-PCR using specific primers for the N (P01 and P02) and E (P03 and P04) gene. All product bands were visualized and quantified using Kodak 1D Imaging Systems and relative mRNA expression was estimated by normalization with β-actin control. The signal intensity of the control vector was considered 100%.

*Western Blot*

Cells were harvested at the indicated time points, washed once with cold PBS and lysed in lysis buffer (PBS, 0.01% EDTA, 1% Triton) containing protease inhibitor cocktail (Roche, USA). Total proteins were separated by 12% SDS-polyacrylamide gel and transferred onto a nylon membrane and incubated with anti-flag (Sigma, USA) monoclonal antibodies, followed by incubation with peroxidase-conjugated immunopure goat anti-mouse secondary antibody (Pierce, USA). The bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA). Then, densitometric scanning of the X-ray film was performed and protein expression level was determined after normalization with β-actin using Kodak 1D Imaging Systems. The signal intensity of the control vector was considered 100%.

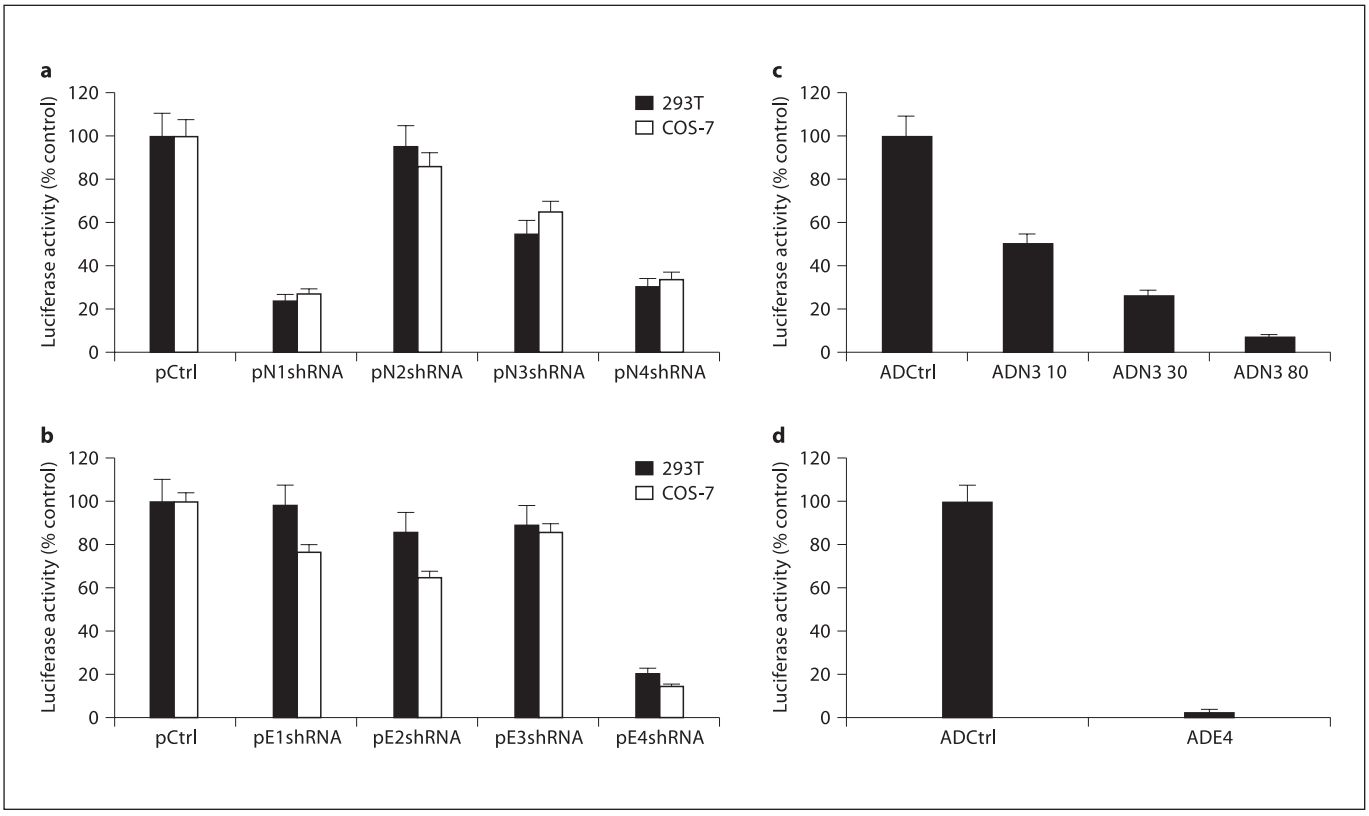


**Fig. 2.** Determination of effects of shRNA and adenovirus-delivered shRNA in transfected cells. 293T cells and COS-7 cells were co-transfected with pLucF-N and pCtrl, pN1shRNA, pN2shRNA, pN3shRNA, pN4shRNA, respectively (**a**). 293T cells and COS-7 cells were co-transfected with pLucF-E and pCtrl, pE1shRNA, pE2shRNA, pE3shRNA, pE4shRNA, respectively (**b**). A random sequence-scrambled shRNA-expressing plasmid pCtrl was used as control. **c** COS-7 cells were infected with ADN3 at the multiplicity of infection (MOI) 10, 30, and 80 after being transfected with pLucF-N plasmid during 24 h. **d** COS-7 cells were infected with ADE4 after transfection with pLucF-E plasmid during 24 h. A random sequence-scrambled shRNA-expressing adenovirus ADCtrl was used as control. Effects of shRNA and adenovirus-delivered shRNA on reporter gene expression were determined by analysis of luciferase activities in cells after being transfected with shRNA.

## Results

*Effects of Specific shRNA Treatment on the Expression of Luciferase Fusion Genes*

To efficiently screen shRNA molecules, selected targeting DNA sequences were fused in frame with that of luciferase gene, in which luciferase activity was supposed to represent the level of N or E mRNA expression. Cells were co-transfected with pLucF-N or pLucF-E and eight single shRNA expression vectors, respectively. Luciferase activities were then determined from both transfected 293T cells and COS-7 cells. Result showed that N1shRNA, N3shRNA, N4shRNA and E4shRNA strongly inhibited luciferase activities by 76, 45, 69 and 79%, respectively, comparing to that of random sequence-scrambled shRNA control (fig. 2a, b) in 293T. Similar results were obtained in COS-7 cells (fig. 2a, b). These results indicated that the four shRNAs can effectively degrade the fusion mRNA of N-luciferase or E-luciferase.

*Suppression of N and E RNA Expression Level by shRNAs*

To determine the effects of shRNA on RNA expression level, total RNAs isolated from transfected cells were used as templates to synthesize cDNA. Specific mRNA was amplified by RT-PCR using gene-specific primers for N (fig. 3a, c), or E (fig. 3b, d), and β-actin was used as an endogenous control. PCR products were detected on agarose gel electrophoresis, visualized under UV light and quantified using Kodak 1D Imaging Systems. Relative

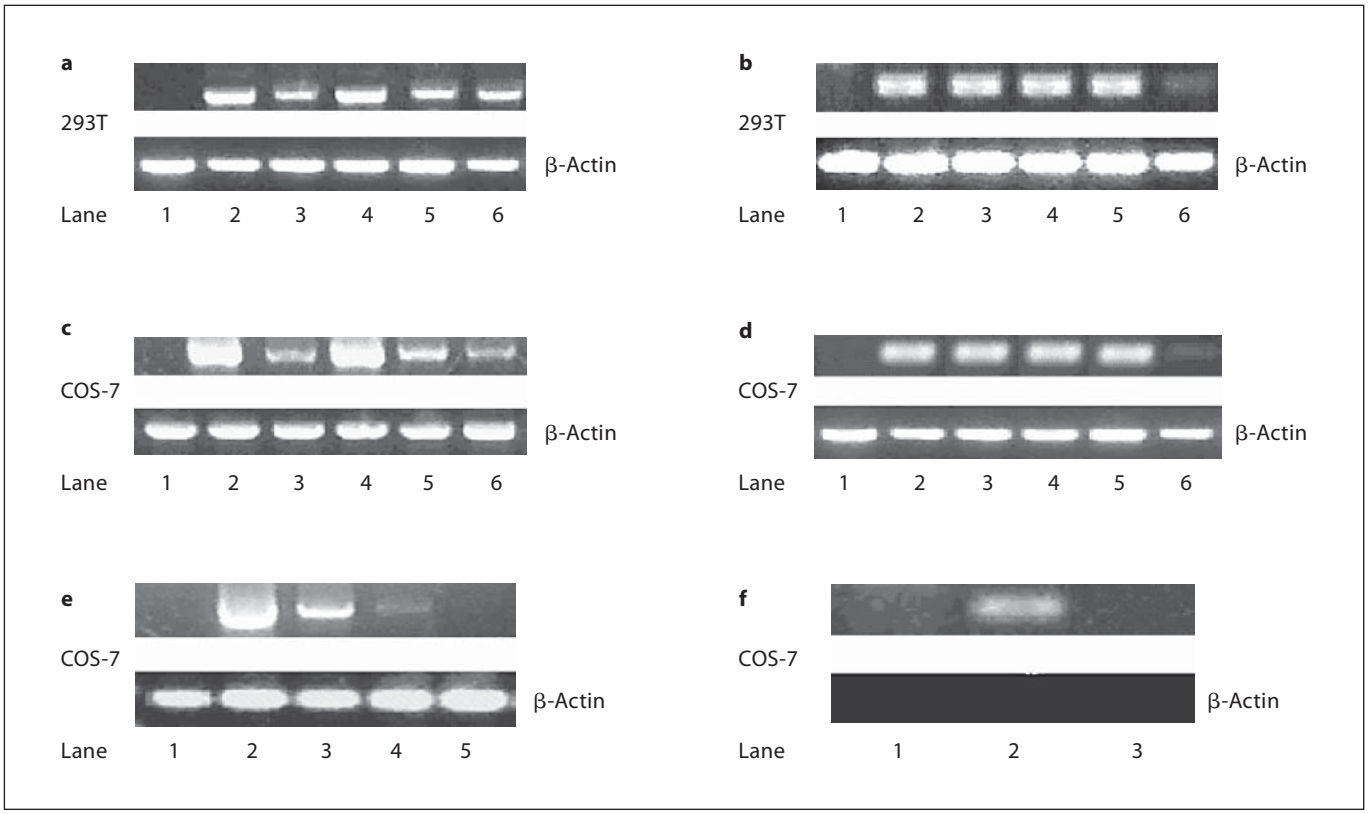


**Fig. 3.** Effects of shRNA and adenovirus-delivered shRNA on level of RNA expression determined by semiquantitative RT-PCR analysis. **a** 293T cells without transfection (lane 1), or co-transfected with pCMV-N and pCtrl (lane 2), pCMV-N and pN1shRNA (lane 3), pCMV-N and pN2shRNA (lane 4), pCMV-N and pN3shRNA (lane 5), and pCMV-N and pN4shRNA (lane 6). **b** 293T cells without transfection (lane 1), or co-transfected with pCMV-E and pCtrl (lane 2), pCMV-E and pE1shRNA (lane 3), pCMV-E and pE2shRNA (lane 4), pCMV-E and pE3shRNA (lane 5), pCMV-E and pE4shRNA (lane 6). **c** COS-7 cells without transfection (lane 1), or co-transfected with pCMV-N and pCtrl (lane 2), pCMV-N and pN1shRNA (lane 3), pCMV-N and pN2shRNA (lane 4), pCMV-N and pN3shRNA (lane 5), and pCMV-N and pN4shRNA (lane 6). **d** COS-7 cells without transfection (lane 1), or co-transfected with pCMV-E and pCtrl (lane 2), pCMV-E and pE1shRNA (lane 3), pCMV-E and pE2shRNA (lane 4), pCMV-E and pE3shRNA (lane 5) pCMV-E and pE4shRNA (lane 6). **e** COS-7 cells were infected with ADN3 at the multiplicity of infection (MOI) 10 (lane 3), 30 (lane 4), and 80 (lane 5) 24 h after being transfected with pLucF-N plasmid. **f** COS-7 cells were infected with ADE4 (lane 3) after being transfected with pLucF-E plasmid during 24 h. ADCtrl (**e** lane 2 and **f** lane 2) and untreated cells (**e** lane 1 and **f** lane 1) were negative controls. To determine effects of shRNAs on viral RNA expression, total RNAs isolated from transfected cells were used as templates to synthesize cDNA. Specific mRNA was amplified by RT-PCR using specific primers for the N gene (fig. 3a, c, e), the E gene-specific primers (fig. 3b, d, f), and the β-actin gene (as an endogenous control). PCR products were detected on agarose gel electrophoresis and visualized under UV light.

mRNA expression was determined by normalization with β-actin. The signal intensity of the control vector was considered as 100%. Results indicate that the levels of N gene mRNA were significantly decreased by 65, 45, and 60% after the treatment of N1shRNA (fig. 3a, lane 3), N3shRNA (fig. 3a, lane 4), N4shRNA (fig. 3a, lane 5) in 293T, respectively. The levels of E mRNA were also decreased by 89% after the treatment of E4shRNA (fig. 3b, lane 3), but not by that of Silencer-2.1-U6 vector (fig. 3a, lane 2, fig. 3b, lane 2) or untreated cells (fig. 3a, lane 1, fig. 3b, lane 1). Similar results were also obtained in COS-7 cells under the same conditions of shRNA treatments (fig. 3c, d). The levels of N mRNA were dramatically reduced by 81, 65, and 75% in COS-7 cells after the treatment of N1shRNA (fig. 3c, lane 2), N3shRNA (fig. 3c, lane 3) and N4shRNA (fig. 3c, lane 4), respectively. The levels of E mRNA in COS-7 cells were also reduced by 91% after the treatment of E4shRNA (fig. 3d, lane 3), but

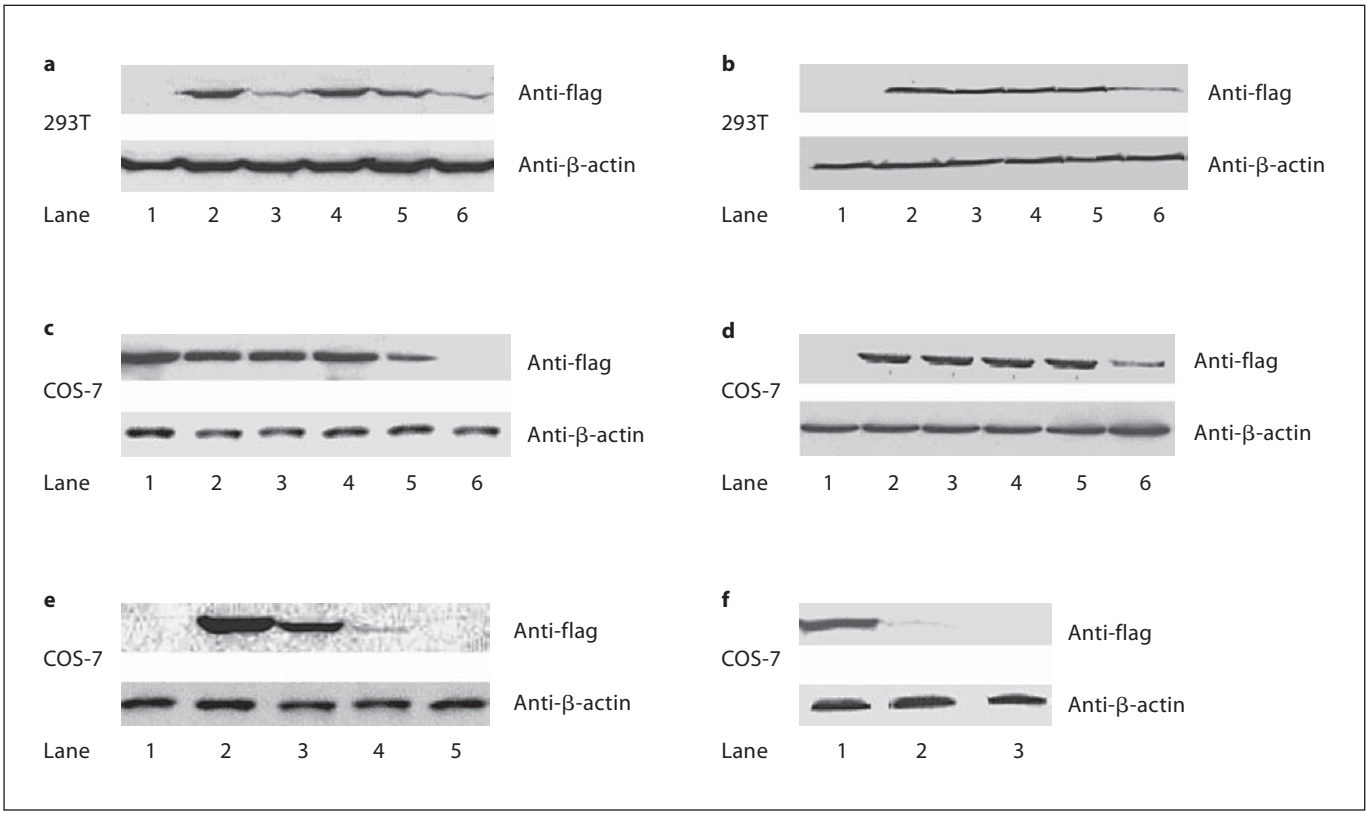


**Fig. 4.** Effects of shRNA and adenovirus-delivered shRNA on protein expression determined by Western blot. **a** 293T cells were co-transfected with pCMV-N and pCtrl (lane 2), pCMV-N and pN1shRNA (lane 3), pCMV-N and pN2shRNA (lane 4), pCMV-N and pN3shRNA (lane 5), and pCMV-N and pN4shRNA (lane 6). 293T cells without transfection were used as controls (lane 1). **b** 293T cells were co-transfected with pCMV-E and pCtrl (lane 2), pCMV-E and pE1shRNA (lane 3), pCMV-E and pE2shRNA (lane 4), pCMV-E and pE3shRNA (lane 5), pCMV-E and pE4shRNA (lane 6). 293T cells without transfection were used as controls (lane 1). **c** COS-7 cells were co-transfected with pCMV-N and pCtrl (lane 1), pCMV-N and pN1shRNA (lane 2), pCMV-N and pN2shRNA (lane 3), pCMV-N and pN3shRNA (lane 4), and pCMV-N and pN4shRNA (lane 5). COS-7 cells without transfection were used as controls (lane 6). **d** COS-7 cells were co-transfected with pCMV-E and pCtrl (lane 2), pCMV-E and pE1shRNA (lane 3), pCMV-E and pE2shRNA (lane 4), pCMV-E and pE3shRNA (lane 5), pCMV-E and pE4shRNA (lane 6). COS-7 cells without transfection were used as controls (lane 1). **e** COS-7 cells were infected with ADN3 at the multiplicity of infection (MOI) 10 (lane 3), 30 (lane 4), and 80 (lane 5) after being transfected with pLucF-N plasmid during 24 h. **f** COS-7 cells were infected with ADE4 (lane 2) after being transfected with pLucF-E plasmid during 24 h. ADCtrl lane 2 (**e**) and lane 1 (**f**) and untreated cells lane 1 (**e**) and lane 3 (**f**) were negative controls.

not by that of pSilencer-2.1-U6 vector (fig. 3c, lane 1, fig. 3d, lane 2) or untreated cells (fig. 3c, lane 5, fig. 3d, lane 1).

*Inhibition of N and E Protein Expression by shRNA Treatment*

To determine whether shRNAs specifically reduce N or E protein expression, Western blot was applied to analyze the levels of flag-tagged N and E protein in 293T (fig. 4a, b) and COS-7 (fig. 4c, d) cells using anti-flag antibody. The results showed that the decrease level of proteins was correlated to the RT-PCR assays in both cell lines.

*Enhanced shRNA Effect by Adenoviral-Mediated Gene Delivery*

To enhance the effectiveness of shRNA delivery and investigate the therapeutic feasibility of shRNA, we generated shRNA-expressing recombinant adenovirus ADN3, ADE4, ADN1, and ADN4 (data not shown), which target different locations of both N and E gene. We also generated a negative control which expresses random se-

quence-scrambled shRNA (ADCtrl). COS-7 cells were infected with each shRNA-expressing adenovirus at the multiplicity of infection (MOI) of 30. To investigate whether the adenovirus-delivered small hairpin RNA can enhance the effectiveness of shRNA, we chose shRNAN3 that had the weakest effect in the inhibition of the N gene expression among the four shRNAs. ADN3 were transduced at the MOI of 10, 30, and 80 to test the dose-dependent delivery efficiency. Cells were harvested day 3 postinfection. Luciferase activity was determined from those transfected cells (fig. 2c, d). Transduction of the shRNA-expressing adenoviruses dose-dependently reduced the expression of their target genes. The target mRNAs and proteins were analyzed by RT-PCR (fig. 3e, f) and Western blot analysis (fig. 4e, f) in parallel, results indicated that further reduction was observed after transduction of shRNA-expressing adenoviruses comparing to that transfection of shRNA-expressing plasmids.

## Discussion

SARS is a newly emerging infectious disease. It is usually characterized by fever, dry cough, myalgia, and mild sore throat, which progresses to a typical pneumonia. Over 8,000 SARS cases and 770 SARS-related deaths were reported to WHO from over 26 countries around the world (http://www.who.int/csr/sars/country/en). SARS is dangerous for its high morbidity and mortality rates. SARS is caused by a newly identified virus within the family *Coronaviridae*. This virus has been designated as the SARS coronavirus (SARS-CoV).

At present, several potential SARS therapies are under development, such as vaccines [33, 34] and interferons [35, 36]. The use of RNAi technology in the therapy of SARS would be advantageous in its specificity for the target gene with minimized side effects. It has the potential to prove useful as novel treatments for virus-induced diseases such as HIV-1, although the problem of how to efficiently deliver small interfering RNA expression vectors, or the small interfering RNAs themselves to target host cells, remains to be resolved.

The interference of SARS-CoV genes expression induced by the use of siRNA duplexes is transient. And because of the high sequence specificity of siRNA-mediated RNA degradation, antiviral efficacy of siRNA directed to viral genome will be largely limited by emergence of escape variants resistant to siRNA due to high mutation rates of virus, especially RNA viruses such as poliovirus, hepatitis C virus and SARS-CoV. The problem can be circumvented by targeting multiple essential genes of the viruses and increasing the delivery efficiency of siRNAs. Among all the approaches the most perhaps desirable one is choosing the adenoviral vector expression system. Our results have demonstrated that inhibition of SARS-CoV genes can be achieved by adenovirus-delivered siRNAs.

In our studies, we have examined gene inhibition by the adenoviral system in COS-7 cells. It is possible that this approach may be applicable to other types of cells as well, especially primary cells that have only a limited lifespan and be resistant to transfection. The high efficiency of adenoviral infection of a variety of host cell types may render this approach a convenient method to achieve gene inhibition through cellular expression of shRNAs. Also it provides the possibility that we apply this technique in animal model in our further investigations.

In conclusion, we developed a simple shRNA delivery strategy by combination of well-defined U6 promoter and conventional pAdEasy-1 adenovirus system. Our results demonstrate significant inhibition of viral genes can be achieved in mammalian cells. Since many cell types can be efficiently infected by adenovirus, recombinant adenoviruses could serve as an alternative powerful tool for delivery of shRNA and for gene suppression, especially when the targeted cells are resistant to transfection by DNA or RNA. In addition, our findings also provide solid evidence for development of an anti-SARS-CoV therapy using adenovirus-delivered small hairpin RNA, which may enhance anti-SARS-CoV efficacy and overcome drug resistance. With availability of high titers of adenoviruses and uniform and rapid infection, this technology will have a foreseeable wide application both in experimental biology and molecular medicine.

## Acknowledgments

This work was supported by research grants from the National Natural Science Foundation of China to J. Wu (No. 30470087 and No. 30570070) and from the Ministry of Education of China to J. Wu (No. 20040486037).

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