

RESEARCH ARTICLE

siRNA targeting the Leader sequence of SARS-CoV inhibits virus replication

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SARS-CoV (the SARS-Associated Coronavirus) was reported as a novel virus member in the coronavirus family, which was the cause of severe acute respiratory syndrome. Coronavirus replication occurs through a unique mechanism employing Leader sequence in the transcripts when initiating transcription from the genome. Therefore, we cloned the Leader sequence from SARS-CoV(BJ01), which is identical to that identified from SARS-CoV(HKU-39849), and constructed specific siRNA targeting the Leader sequence. Using EGFP and RFP reporter genes fused with the cloned SARS-CoV Leader sequence, we demonstrated that the siRNA targeting the Leader sequence decreased the mRNA abundance and protein expression levels of the reporter genes in 293T cells. By stably expressing the siRNA in Vero E6 cells, we provided data that the siRNA could effectively

and specifically decrease the mRNA abundance of SARS-CoV genes as analyzed by RT-PCR and Northern blot. Our data indicated that the siRNA targeting the Leader sequence inhibited the replication of SARS-CoV in Vero E6 cells by silencing gene expression. We further demonstrated, via transient transfection experiments, that the siRNA targeting the Leader sequence had a much stronger inhibitory effect on SARS-CoV replication than the siRNAs targeting the Spike gene or the antisense oligodeoxynucleotides did. This report provides evidence that targeting Leader sequence using siRNA could be a powerful tool in inhibiting SARS-CoV replication.

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Introduction

SARS-CoV (the SARS-Associated Coronavirus), which is the pathogen of severe acute respiratory syndrome (SARS),^{1,2} was reported to be a novel member in the coronavirus family.^{3–6} Coronaviruses belong to a family of enveloped viruses containing a single-stranded plus-sense RNA genome about 30 kb in length with a 5' cap structure and a 3' polyadenylation tract.⁷ In addition to the genomic RNA, virus-infected cells possess subgenomic mRNAs that form a 3' coterminal nested set with the genome. The genomic and the subgenomic mRNAs of coronaviruses include an identical 5' Leader sequence and common 3'-ends, which is a unique characteristic in coronavirus and arterivirus replication.⁸ In the process of coronavirus replication, the Leader sequence (from the subgenome) is fused to the mRNA body sequence. The mechanism by which these subgenomic mRNAs are made is not fully understood. However, recent evidence indicates that transcription-regulating sequences (TRSs) at the 5'-end of each gene

represent signals that regulate the discontinuous transcription of subgenomic mRNAs.^{9,10} Nevertheless, the Leader sequence and the TRSs play critical roles in the gene expression of coronavirus during its replication.^{9,10}

By analysis and comparison of the genome sequences of SARS-CoV^{11–14} and other coronaviruses, authors predicted^{11,12} and confirmed¹⁵ a putative 5' Leader sequence with similarity to the conserved coronavirus core Leader sequence, 5'-CUAAAC-3',^{9,10} at the 5'-end of the genome. Putative conserved TRS sequences AAAC GAAAC were determined through manual alignment of sequences upstream of potential initiating methionine codons with the region of the coronavirus genome sequence containing the Leader sequence. These predictions suggested that SARS-CoV possessed a similar genome structure and underwent similar replication processes to those of other typical coronaviruses.^{11,12} Therefore, it is of interest to search for a way to block virus replication based on this common feature of the coronavirus.

RNAi technology¹⁶ has been approved as a potential tool¹⁷ to inhibit infectious virus in host cells because siRNA can target and silence important genes required by the virus.¹⁸ In an attempt to inhibit virus replication, authors have demonstrated that siRNA could effectively inhibit poliovirus and Rous sarcoma virus replication.^{19,20} Furthermore, to block HIV replication and

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infection, siRNAs were designed to target critical genes in the HIV life cycle including p24 (the HIV long-terminal repeat),^{21,22} vif, nef,²³ tat and rev.^{24,25} The results showed a very promising inhibition effect on the virus both in cell culture and in mouse models. Using the same methodology, siRNAs were used to inhibit hepatitis C (HCV) or hepatitis B (HBV) replication by targeting the critical genes required for virus replication.^{26–29} These combined results are very promising since they showed inhibition of viral replication in cell cultures as well as in mouse models.

Recent studies have shown that siRNAs could inhibit gene expression and thereby inhibit the replication of SARS-CoV in cultured cells.^{30–32} However, these reports focused on the specific genes in the SARS-CoV genome, where there would be a high probability of single nucleic acid changes,¹⁴ leading to reduced effectiveness of siRNA targeting. Therefore, choosing a unique sequence in the SARS-CoV genome will be critical for the successful inhibition of the virus. In this report, we propose to target the Leader sequence of SARS-CoV, which is identical in all SARS-CoV and plays a pivotal role in the gene expression of the virus. We demonstrate the siRNA we generated could effectively and specifically silence gene expression and inhibit SARS-CoV replication in cultured mammalian cells. Our data provide a possible way of using gene therapy for SARS.

Results

Cloning of the Leader sequence and design of siRNA

Based on the high homologies between genome sequences of BJ01 SARS-CoV (GenBank accession number AY278488) and mouse hepatitis virus and the identification of the Leader sequence from HKU-39849,^{11,12} we predicted the identical Leader sequence of BJ01 SARS CoV (data not shown). To clone the Leader sequence predicted from BJ01 SARS CoV, we performed 5'-RACE experiments to confirm the Leader sequence. We then used RT-PCR to obtain the cDNA of the transcripts of different genes of BJ01 SARS-CoV from infected Vero E6 cells. After sequencing, we observed the identical Leader sequence upstream of the ATGs in the different genes (Figure 1a, GenBank accession numbers AY539954, AY536757, AY536758, AY536759, AY536760), suggesting that the transcripts of different genes in BJ01 SARS-CoV recruited the identical Leader sequence, which is consistent with the report from HKU-39849.¹⁵ The data indicated that the transcript of the S protein contained the Leader sequence directly upstream of the ATG, while the transcripts of other proteins had different nucleotide bases between the ATG and the conserved Leader sequence (eg M protein has 44 more base pairs) (Figure 1a). Intriguingly, this Leader sequence was highly conserved between different strains of SARS-CoV, while the other sequences coding for the specific proteins such as S, N, M and E contained various mutations between the different strains (Table 1). According to this information, the sequence of CCAACCAACCTCGATCTC was selected and designed as the siRNA target for the proper GC/AT ratio and CC structure. To generate the dsRNA based on this sequence, we employed the U6 promoter vector³³ and constructed a 22 bp siRNA through the

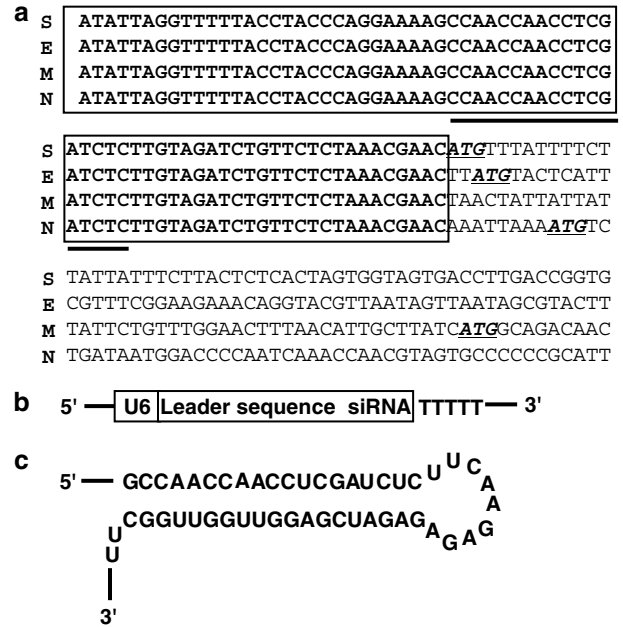


Figure 1 Sequence information for SARS-CoV used for siRNA design. (a) Leader sequence information from different transcripts of the SARS-CoV. The transcripts of SARS-CoV from the infected Vero E6 cells were 5'-RACE and RT-PCR amplified and sequenced. The identical Leader sequences were boxed. The start codons (ATG) for different genes are underlined with italic letters. The targeted sequence in the Leader sequence of SARS-CoV is underlined. The one-step RT-PCR reaction condition was 35 cycles of denaturation (94°C, 30 s), annealing (51°C, 45 s) and extension (72°C: S gene, 1 min; E gene, 25 s; M gene, 45 s; N gene, 1 min 20 s). (b) Schematic of vector for generating siRNA. pBS/U6 promoter, sequence generating siRNA hairpin and transcriptional terminal signal are shown as indicated. (c) Diagram of predicted structure of siRNA from pBS/U6/L-RNAi. UUCAAGAGA was used to generate the hairpin loop, indicated as a cycle.

Table 1 Mutation of SARS CoV

Region	Mutated sites	Strains with the mutations ^a
S	58	94
E	4	12
M	20	58
N	22	30
Leader	1	1

^aTotally, 118 strains reported were searched.

hairpin (Figure 1b). The expected hairpin RNA is depicted in Figure 1c.

Construction of the reporter gene fused with the Leader sequence

To test if the vector-based siRNA we designed could silence the expression of genes with the Leader sequence, we used GFP and RFP as reporter genes. We inserted the Leader sequence of SARS-CoV upstream of the first ATG of GFP after the EF-1 α promoter (Figure 2a, top panel) or RFP after the CMV promoter (Figure 2a, bottom panel) in mammalian expression vectors. As controls, we directly used the GFP or RFP expression vector without the Leader sequence (Figure 2a, middle panels). To show the correct transcription of the mRNAs, we transfected the vectors into 293 cells and performed RT-PCR using

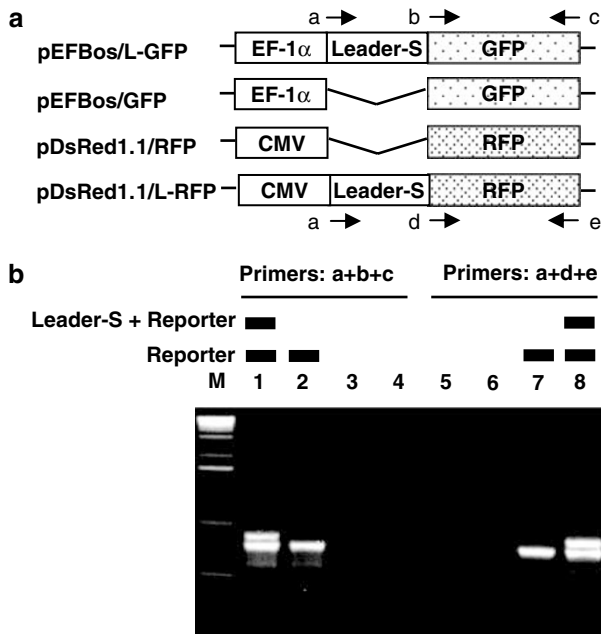


Figure 2 Artificial reporter vectors with and without a Leader sequence. (a) Diagram for the vector design. Leader sequence (labeled as Leader-S in the box) from SARS-CoV was synthesized and inserted upstream of a GFP or RFP reporter gene driven by an EF-1 α or CMV promoter as indicated. a–e are primers described in Materials and methods. (b) RT-PCR results showing the mRNAs from the reporter vectors. 293T cells were transfected with pEFBos/L-GFP (lanes 1 and 5), pEFBos/GFP (lanes 2 and 6), pDsRed1.1/RFP (lanes 3 and 7), pDsRed1.1/L-RFP (lanes 4 and 8). The total RNA was isolated for performance of RT-PCR using the primers as indicated. The predicted bands are listed above the gel. The upper bands (noted as Leader-S+Reporter) are the mRNA from reporter constructs with the Leader sequence of SARS-CoV and the lower bands (noted as Reporter) are from reporter constructs without the Leader sequence. DNA ladder is shown on the left of the gel. The PCR reaction condition was 25 cycles of denaturation (94°C, 30 s), annealing (50°C, 1 min) and extension (72°C, 1 min).

different primers as showed in Figure 2a. The data demonstrate that primers a, b and c amplify two bands in cells transfected with pEFBos/L-GFP, but only one band in cells transfected with the pEFBos/GFP plasmid, while there is no band in cells transfected with pDsRed1.1/L-RFP or pDsRed1.1/RFP, suggesting that the EF-1 α promoter in the vector could produce GFP mRNA with a Leader sequence of SARS-CoV. In contrast, using primers a, d and e, we obtained two bands as predicted in cells transfected with pDsRed1.1/L-RFP (Figure 2b, lane 8) and one band with pDsRed1.1/RFP (Figure 2b, lane 7). The primers a, d and e could not amplify any fragment in cells transfected with pEFBos/L-GFP or pEFBos/GFP (Figure 2b, lanes 5 and 6). These data indicate that we can generate GFP or RFP mRNAs with the SARS-CoV Leader sequences from vectors with either the EF-1 α or CMV promoter.

The RT-PCR results show that the EF-1 α or CMV promoter initiated the transcription of mRNA of GFP or RFP with a SARS-CoV Leader sequence (Figure 2b). We questioned whether these mRNAs could be translated into GFP or RFP proteins. To test this probability, we directly tested the expression of GFP and RFP using a fluorescence microscope in cells transfected with the vectors. The data show that all four vectors could express GFP or RFP properly (Figure 4b), suggesting that the

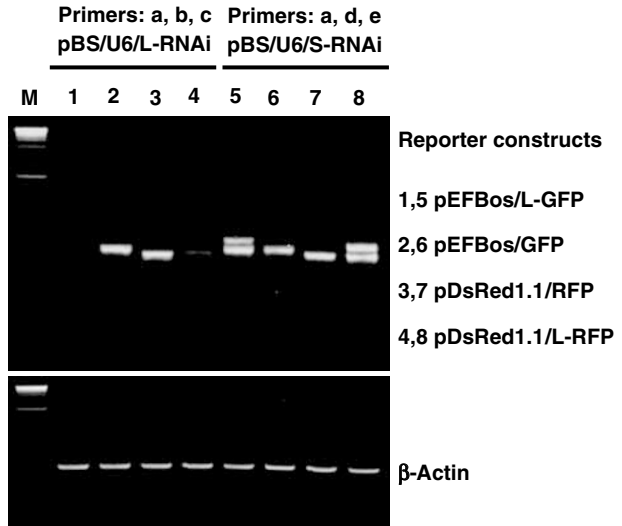


Figure 3 Inhibition of reporter gene expression by siRNA. The reporter constructs as indicated were cotransfected with the pBS/U6/L-RNAi (lanes 1–4) or pBS/U6/S-RNAi (lanes 5–8) into 293T cells. Total RNA was isolated for performance of RT-PCR using the primers as indicated. The comparison of the effect of siRNA should be between lanes 1 and 5, 2 and 6, 3 and 7, and 4 and 8. β -actin was used as an internal control. The PCR reaction conditions were 25 cycles of denaturation (94°C, 30 s), annealing (50°C, 1 min) and extension (72°C, 1 min).

Leader sequence ahead of the ATG of GFP or RFP did not affect the translation of proteins. Therefore, we directly observed the proteins produced by the mRNA with the Leader sequence. These vectors therefore provided us a model to study degradation of mRNAs using a Leader sequence of SARS-CoV targeting by siRNA.

siRNA targeting the Leader sequence silenced the reporter gene expression

To test whether the siRNA could mediate the degradation of mRNA with the Leader sequence from SARS-CoV, we examined the mRNA levels of the reporter genes from each of the four vectors (to generate different mRNAs) cotransfected with pBS/U6/L-RNAi (Figure 3, lanes 1–4), a vector generating siRNA targeting the Leader sequence of SARS-CoV, or an unrelated pBS/U6/S-RNAi plasmid, an siRNA vector targeting the S gene of SARS-CoV³¹ (Figure 3, lanes 5–8). The data show that cotransfection of pBS/U6/L-RNAi, compared to the control vector, significantly decreased the mRNA levels of L-GFP and L-RFP containing the Leader sequence of SARS-CoV (Figure 3, comparing lane 1 to lane 5, or lane 4 to lane 8), while it had no effect on the mRNAs of GFP and RFP without the Leader sequence (Figure 3, comparing lane 2 to lane 6, or lane 3 to lane 7). These data suggest that siRNA targeting the Leader sequence of SARS-CoV specifically led to a decrease in mRNA levels of L-GFP or L-RFP generated by the pEFBos or pDsRed1.1 vectors, implying that siRNA targeting the Leader sequence mediated degradation of mRNA produced by the transfected vectors.

The advantage of using GFP and RFP is that it facilitates the direct observation of the proteins by fluorescence microscopy. To demonstrate that decreased L-GFP mRNA produced less protein, we applied fluorescence microscopy to view the cells cotransfected with pEFBos/

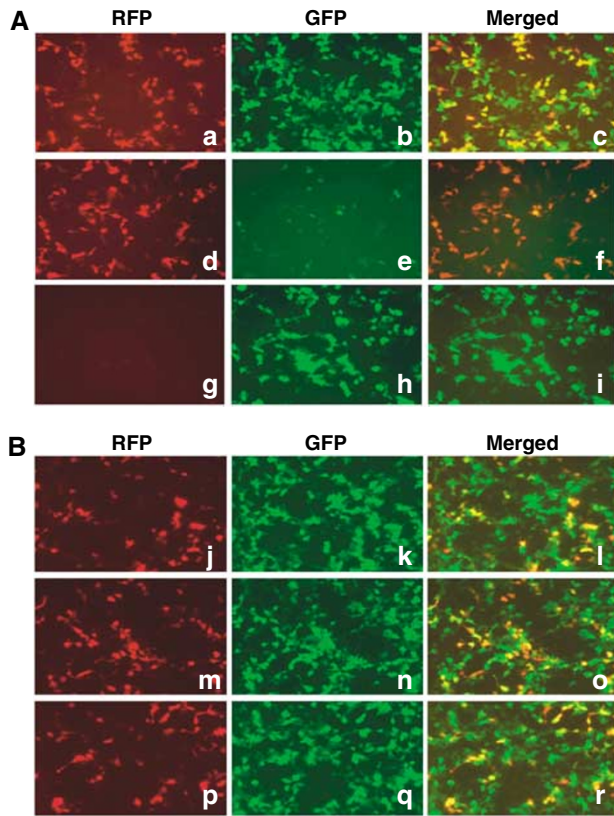


Figure 4 Fluorescence microscopy observation of reporter gene expression in transfected cells. 293T cells were cotransfected with the reporter constructs and the pBS/U6/L-RNAi (A) or pBS/U6/S-RNAi (B) vector. The reporter constructs used are a, b and c, and j, k and l: pEFBos/GFP and pDsRed1.1/RFP; d, e and f, and m, n and o: pEFBos/L-GFP and pDsRed1.1/RFP; g, h and i, and p, q and r: pDsRed1.1/L-RFP and pEFBos/GFP. The same field was observed by red color (excitation, 510–560 nm; emission, 590 nm) and green color (excitation, 450–490 nm; emission, 520 nm) and the two color pictures were merged in the right panel. Note that pBS/U6/L-RNAi significantly inhibited reporter gene expression (e and g).

L-GFP and pDsRed1.1/RFP (internal control) with pBS/U6/L-RNAi (Figure 4a) or pBS/U6/S-RNAi (negative control, Figure 4b). The data show that both GFP driven by pEFBos/L-GFP and RFP driven by pDsRed1.1/RFP were expressed strongly in the cells in the presence of pBS/U6/S-RNAi, a vector generating siRNA targeting the S gene of SARS-CoV³¹ (Figure 4b), while only RFP was strong but GFP was weak in the cells (transfected with pEFBos/L-GFP and pDsRed1.1/RFP) in the presence of pBS/U6/L-RNAi (Figure 4Ad–f), suggesting that the siRNA targeting the Leader sequence blocked GFP expression from the vector where the GFP gene was linked to the Leader sequence. Parallel to L-GFP, we also investigated L-RFP expression in cells cotransfected with pDsRed1.1/L-RFP and pEFBos/GFP (internal control) with pBS/U6/L-RNAi (Figure 4A) or pBS/U6/S-RNAi (Figure 4B). The data confirmed that RFP was dramatically decreased in the cells transfected with pDsRed1.1/L-RFP and pEFBos/GFP in the presence of pBS/U6/L-RNAi (Figure 4Ag), while GFP protein was expressed at similar levels in the same cells (Figure 4Ah), suggesting that the siRNA inhibited the expression of RFP from pDsRed1.1/L-RFP plasmid, where the Leader sequence of SARS-CoV was upstream of the RFP, but did not effect

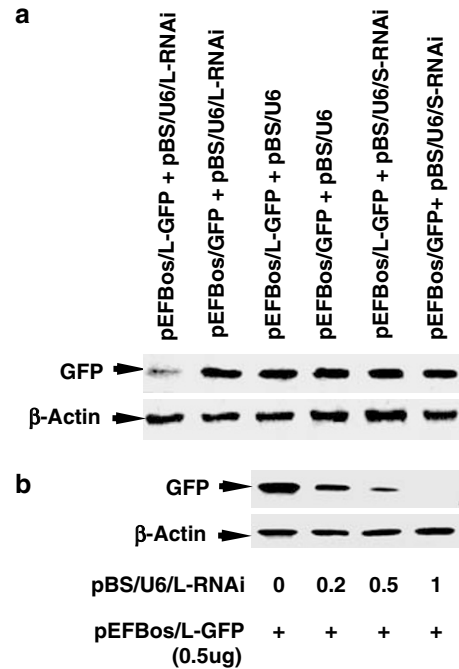


Figure 5 Western blot analysis of GFP protein expression in transfected cells. (a) pEFBos/L-GFP or pEFBos/GFP vector was cotransfected with pBS/U6/L-RNAi, pBS/U6 or pBS/U6/S-RNAi in Vero E6 cells as indicated. The cells were lysed and subjected to Western blot using anti-GFP antibody. β -actin was used as internal control to indicate even transfection efficiency and loading. (b) pEFBos/L-GFP (0.5 μ g) were cotransfected with the indicated increasing amounts of pBS/U6/L-RNAi in Vero E6 cells. Western blot was employed to show the expression of GFP protein. Note that the GFP protein decreased dramatically with increasing amounts of pBS/U6/L-RNAi used, while the β -actin level remained stable.

the expression of GFP driven by pEFBos/GFP in the same cells. Importantly, pBS/U6/L-RNAi did not have any affect on the expression of either RFP or GFP proteins driven by the constructs without the Leader sequence of SARS-CoV (Figure 4Aa–c). As an additional control, pBS/U6/S-RNAi was shown to have no effect on the expression levels of RFP and GFP proteins (Figure 4b). All the data indicate that the siRNA could specifically and effectively block the expression of the reporter genes fused with the Leader sequence of SARS-CoV.

To measure the protein levels of GFP in the cells, we employed Western blot analysis. The results show that GFP protein levels were decreased significantly in the presence of a pBS/U6/L-RNAi vector (Figure 5a, lane 1), while they did not change in the other situations (Figure 5a, lanes 2–6). These data are consistent and supportive of those observed by fluorescence microscopy. Furthermore, to determine whether the inhibition of the siRNA on reporter gene expression is dependent on the amount of the siRNA, we used three different dosages of the pBS/U6/L-RNAi plasmid (0.2, 0.5 and 1.0 μ g) cotransfected with 0.5 μ g of pEFBos/L-GFP. Western blot analysis suggests that the GFP protein levels decreased gradually with increasing amounts of pBS/U6/L-RNAi plasmid (Figure 5b). Intriguingly, 1.0 μ g of pBS/U6/L-RNAi plasmid almost totally inhibited the expression of the reporter gene, indicating that the siRNA effectively silenced the expression of the reporter gene with the Leader sequence from SARS-CoV.

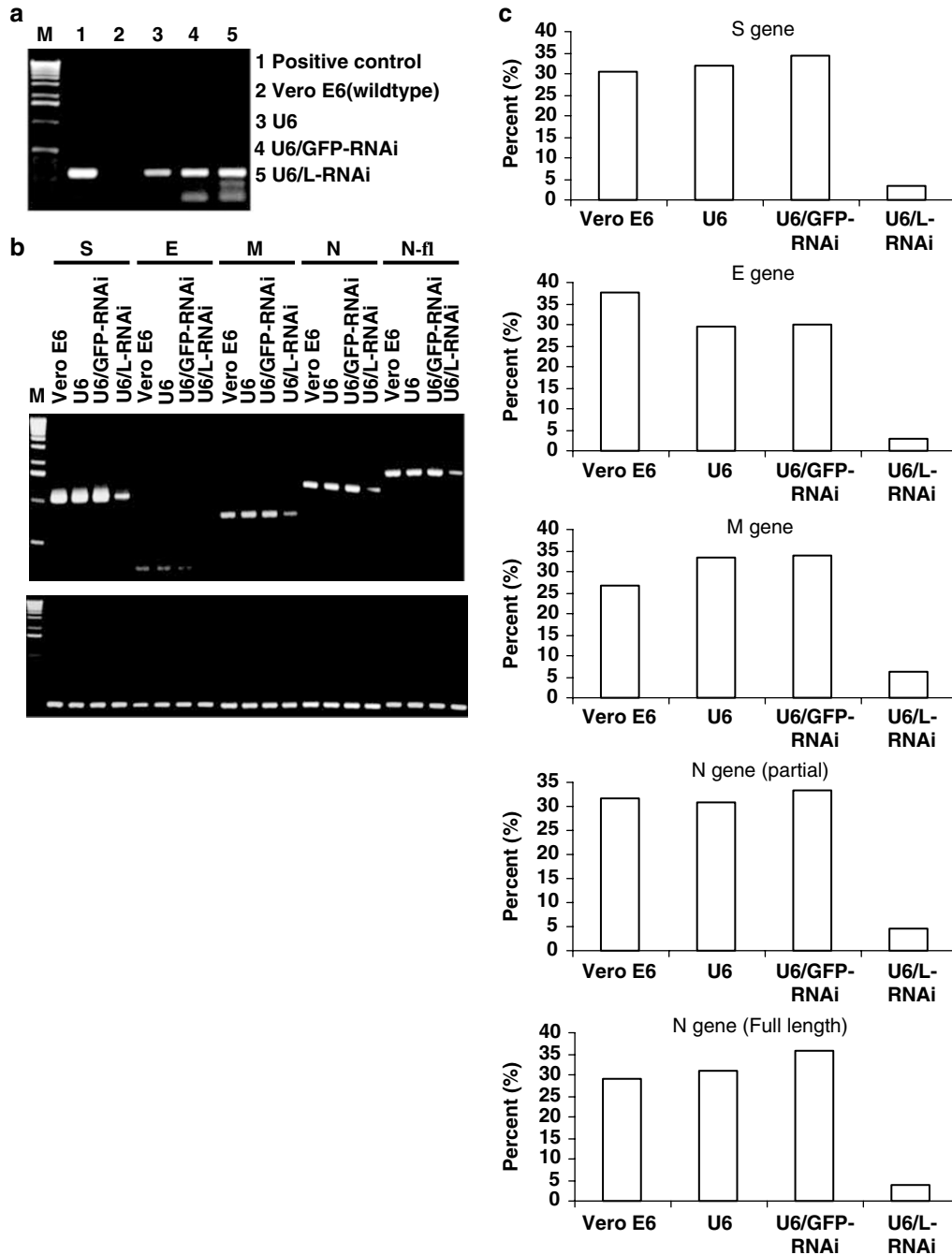


Figure 6 Inhibition of SARS-CoV gene expression by siRNA in Vero E6 cells. (a) Establishment of stable cell lines with the siRNA. Vero E6 cells were transfected with the indicated plasmids. The cells were selected with G418 and the clones were examined by PCR with specific primers described in Materials and methods. The cells were named using the recombinant U6, U6/GFP-RNAi or U6/L-RNAi, respectively. The positive control was from the cells transiently transfected with pBS/U6/L-RNAi. (b) L-RNAi inhibits different gene expression of SARS-CoV in stable cell lines detected by RT-PCR. The PCR reaction condition was cycles (S gene, 30; E gene, 27; M gene, 27; N gene, 25; N-fl, 25) of denaturation (94°C, 30 s), annealing (51°C, 45 s) and extension (72°C: S gene, 1 min; E gene 25 s; M gene 45 s; N gene, 1 min 20 s; N-fl, 1 min 45 s). The indicated stable cell lines in 60 mm dishes were infected with SARS-CoV (BJ01) at 1×10^6 PFU for 2 h and cultured for 24 h. The total RNA of the cell lysates was used for the RT-PCR. β -actin was used as internal control for same PCR cycles and loading. The different gene fragments are labeled as S, E, M, N and N-fl, respectively. Note that the amounts of the PCR products were dramatically decreased in the stable cell line with U6/L-RNAi. (c) Quantitative analysis of PCR products. All the bands were quantitated by ImageQuant5.1 software (Amersham-Pharmacia's Biotech). The percentages of the band densities were presented.

Inhibition of SARS-CoV by pBS/U6/L-RNAi in Vero E6 cells

To study the inhibition of SARS-CoV by the designed siRNA (pBS/U6/L-RNAi), we subcloned the U6/L-

RNAi cassette into pcDNA3, which contained Neo gene, and selected stable cell clones for constant expression of siRNA by G418 selection. As controls, we selected cell clones with pcDNA3/U6 or pcDNA3/U6/GFP-RNAi.

We used PCR to verify the clones containing the U6/L-RNAi cassette (Figure 6a). The cell clones were named Vero E6 (U6/L-RNAi), Vero E6 (U6) and Vero E6 (U6/GFP-RNAi), respectively.

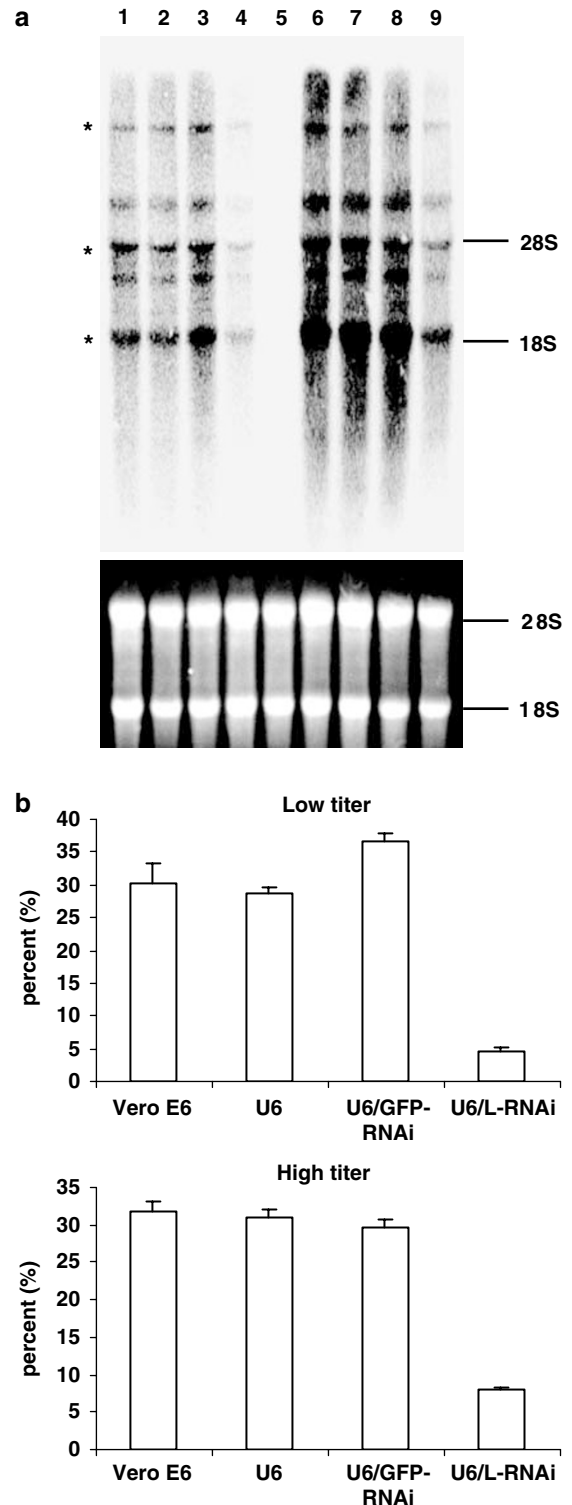
To demonstrate if the siRNA could reduce mRNA abundance of different genes from SARS-CoV in the cultured cells, we infected the cell clones with SARS-CoV (BJ01) with 1×10^6 PFU/well for 2 h. After 24 h, we harvested the cells and detected the different gene transcripts using RT-PCR. As shown in Figure 6b, SARS-CoV-infected Vero E6, Vero E6 (U6) and Vero E6 (U6/GFP-RNAi) cells showed equal amounts of mRNAs, whereas SARS-CoV-infected Vero E6 (L-RNAi) cells showed a significant decrease of mRNAs (Figure 6b). To quantify the results, we scanned the densities of the bands and the data presented in Figure 6c. These figures clearly demonstrate that the siRNA targeting the Leader sequence significantly inhibited gene expression of all the detected genes including the S, E, M and N genes of SARS-CoV.

To further confirm the RT-PCR results, we performed Northern blot analysis using total RNA isolated from infected cells (two titers of SARS-CoV infection). We successfully detected five major transcripts (Figure 7a), which was consistent with the expected result.¹¹ We quantitated these five transcripts using the Phosphor-Imager program (Figure 7b). The data indicate that the mRNAs from cells with the siRNA targeting the Leader sequence were significantly decreased (Figure 7a, comparing lane 4 to lanes 1, 2 and 3, and lane 9 to lanes 6, 7 and 8) as expected. Taken together, these data demonstrate that the siRNA we designed could effectively decrease the expression of genes of SARS-CoV in cultured cells.

To determine whether the vector-based siRNA could efficiently prevent SARS-CoV replication and infection, we plated the cells in a 24-well plate at a density of 1×10^5 cells/well and infected the cells with 1×10^6 PFU of SARS-CoV (BJ01). After 72 h of culture, to demonstrate the inhibition of SARS-CoV, we stained the cells with crystal violet (Figure 8a). The results show that the cells infected with SARS-CoV exhibited greater resistance to virus infection, because the Vero E6 (L-RNAi) cells were stained as strongly as the noninfected cells were (Figure 8a, plates 1 and 3), while the Vero E6, Vero E6 (U6) and Vero E6 (U6/GFP-RNAi) cells stained equally weakly (plates 2, 4 and 5) demonstrating cell destruction.

Figure 7 SARS-CoV gene expression patterns in Vero E6 cells. (a) Northern blot of different genes of SARS-CoV in transfected cells. The total RNA from the indicated cells infected with SARS-CoV was subjected to Northern blot analysis with 32 P-labeled probes amplified from SARS-CoV cDNA (BJ01). 28S and 18S ribosome RNAs were used as control for even loading. Lanes 1–4 represent the indicated cells infected with low titers (1×10^6 PFU) of SARS-CoV and lanes 6–9 represent higher titers (2×10^7 PFU) of virus infection. Lane 5 presents the cells without infection. The indicated stable cell lines were infected with SARS-CoV (BJ01) for 2 h and cultured for 24 h. (b) Quantitative presentation of the gene expression patterns of SARS-CoV. Three bands indicated as asterisk in each treatment were quantitated by ImageQuant5.1 software (Amersham-Pharmacia's Biotech). The band densities were presented as percentages. The three band percentages were averaged. The bars denote the standard error of the mean. The results from low or high titer virus infection were presented separately as indicated.

Furthermore, we viewed the cells for morphological changes (Figure 8b). Significant cytopathic effects were seen in the plates with the Vero E6 wild-type cell, Vero E6 (U6) cells and Vero E6 (U6/GFP-RNAi) cells (Figure 6e, 2, 4, 5), while the noninfected cells (Figure 8b, 1) and SARS-CoV-infected Vero E6 (U6/L-RNAi) cells (Figure 8b, 3) maintained their normal morphology. To measure



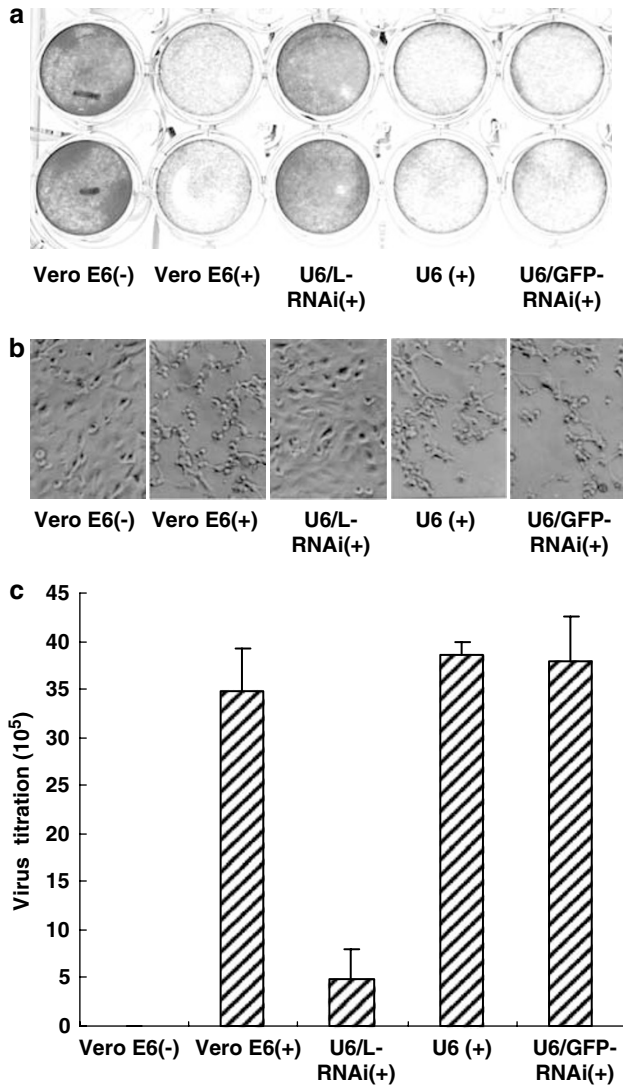


Figure 8 SARS-CoV infection in Vero E6 cells in the presence of stable expression of siRNAs. (a) Cell staining. The indicated cells in 24-well plates were infected with 1×10^6 PFU of SARS-CoV per well for 2 h and cultured for 72 h. The cells were stained with crystal violet. The staining represents normal cells and no staining represents lysed cells by the SARS-CoV. (+) indicates the cells infected with SARS-CoV. (-) indicates normal cultured cells. Duplicate plates are presented. (b) Cell morphology observation. The indicated cells (same treatment with SARS-CoV as D) were subjected to microscopy for the observation of cell morphology. (+) indicates the cells infected with SARS-CoV. (-) indicates normal cultured cells. (c) SARS-CoV titration in the supernatant medium of the infected cells. The same titers of SARS-CoV were used to infect the cells as indicated at the same conditions in (a and b). The virus titrations were determined for the supernatant medium using 96-well plates. The titrations were presented as PFU. Three independent assays were performed and the results were presented as average plus standard error. (+) indicates cells infected with SARS-CoV, (-) indicates cells without SARS-CoV infection.

the virus levels in the infected cells, we used real-time PCR to determine MOI of the virus. The data show that only the cells with the Leader sequence siRNA had a low virus titer, while the other cells expressed high titers of virus (Figure 8c). These data demonstrate that the siRNA we designed dramatically decreased the replication of SARS-CoV in cultured Vero E6 cells.

siRNA targeting the Leader sequence is more effective to inhibit SARS-CoV replication than the siRNAs targeting the S gene or antisense oligodeoxynucleotides

We previously reported that siRNAs targeting the S gene could inhibit SARS-CoV replication,³¹ therefore, we questioned whether the siRNA targeting the Leader sequence was more effective than targeting a specific gene. To compare the effectiveness, we used the same amounts of plasmids to transfect Vero E6 cells and measured the virus titers in the supernatant of the cells infected by the same amounts of SARS-CoV. The data (Figure 9a) show that the virus titer was 4.4×10^6 PFU in the infected cells with U6/GFP-RNAi plasmid, while the virus titers decreased to 4.2×10^5 , 4.8×10^5 and 7.8×10^4 PFU in the cells with U6/S-RNAi1, U6/S-RNAi2 and U6/L-RNAi plasmids, respectively, indicating that the siRNAs we designed could inhibit the virus

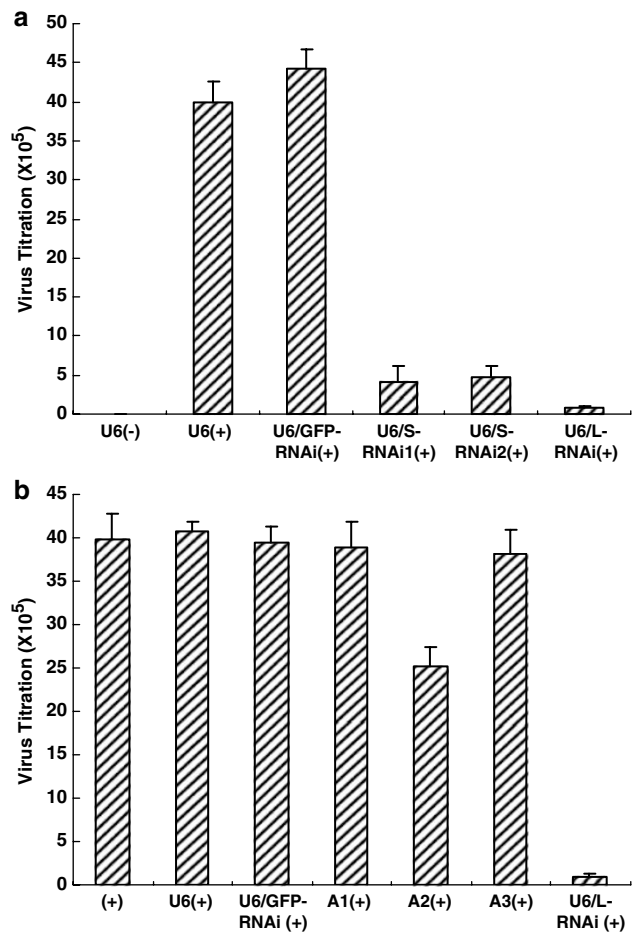


Figure 9 SARS-CoV infection in Vero E6 cells transiently transfected with siRNA vectors or oligodeoxynucleotides. The indicated siRNA plasmids and the antisense oligodeoxynucleotides were transiently transfected into Vero E6 cells in 24-well plates via Lipofectamine 2000. The transfected cells were infected with the 1×10^5 PFU of SARS-CoV per well for 2 h and cultured for 48 h. The virus titers in the cells were evaluated and the titrations were presented as PFU. Three independent assays were performed and the results were presented as average plus standard error. (+) indicates cells infected with SARS-CoV, (-) indicates cells without SARS-CoV infection. (a) Comparison of siRNA targeting the Leader sequence and the Spike gene. (b) Comparison of siRNA targeting to the Leader sequence and antisense oligodeoxynucleotides.

replication dramatically. Interestingly, comparing with the control (U6/GFP-RNAi), the virus titers in cells transfected with U6/S-RNAi1 and U6/S-RNAi2 was 9.4 and 10.9%, while that with U6/L-RNAi was 1.8%, suggesting that U6/L-RNAi had a much stronger inhibitory effect on the virus replication than U6/S-RNAi1 or U6/S-RNAi2 did.

To address whether the antisense has any effect on the virus replication, we generated antisense oligodeoxynucleotides by directly synthesizing oligodeoxynucleotides. We transfected the same amounts of oligodeoxynucleotides as pBS/U6-L-siRNA (1 μ g of DNA each in 24-well plates) in each data culture. The data (Figure 9b) show that one of the antisense oligodeoxynucleotides (A2) could inhibit the virus to 61.9%, while the other two (A1 and A2) had no effect. Again, the pBS/U6-L-siRNA inhibits the virus to 2.2%. These data clearly indicate that targeting Leader sequences could strongly inhibit SARS-CoV replication in Vero E6 cells.

Discussion

SARS has emerged as one of the newest severe infectious diseases threatening global travel and having a significant economic impact on various countries. Caused by a novel coronavirus, SARS cannot be treated by the traditional antiviral drugs, although interferons may be useful in therapy.^{31,34,35} Additional strategies for the control of this disease have been postulated³⁶ with vaccines potentially being the best approach, but vaccine development will take at least one to several years.^{3,37} Even though we can expect vaccine development,^{38,39} alternative therapeutic methods need to be investigated.³⁶ In this report, we investigated the possibility of applying siRNA targeting the Leader sequence of SARS-CoV to treat SARS. We demonstrated that this method was powerful in inhibiting SARS-CoV replication by inhibiting gene expression of the virus in cells. Our report, together with others in HIV, HCV and HBV,^{20,22–26,28,40–42} provide hope of directly blocking virus replication in cells.

SARS-associated coronavirus has been classified as a novel member in the coronavirus family.^{4,11,12} The structure of the virus is similar to the other coronaviruses, which contain S, M, E and N proteins playing important roles in viral entry, replication and pathogenesis.⁴³ The common feature of the coronaviruses is the similar discontinuous transcription of subgenomic mRNAs, regulated by the pretranscribed Leader sequence and the specific TRSs in the genome of the virus.^{9,10} The Leader sequence contributes to the regulation of virus gene expression. Therefore, targeting the Leader sequence present in the mRNA of SARS-CoV structural genes, should be a powerful way to control virus replication. Interestingly, we analyzed all the strains of SARS-CoV identified to date and found that the Leader sequence remained almost identical (see Table 1). With these advantages, we designed the siRNA to target this Leader sequence of SARS-CoV. Our data show that the siRNA could not only block the expression of a reporter gene fused with the Leader sequence but also inhibited the expressions of SARS-CoV genes in Vero E6 cells, and thereafter inhibited the replication of the SARS-

CoV in Vero E6 cells. We proposed that this would be a powerful tool to prevent SARS-CoV infections *in vivo*.

It was previously suggested that coronavirus used the core Leader sequence, 5'-CUAAAC-3'.^{9–12,15} In this report, we cloned the transcripts of the four genes from SARS-CoV BJ01 and confirmed the SARS-CoV Leader sequence as CCAGGAAAAGCCAACCAACCTCGA TCTCTGTAGATCTGTTCTCTAAACGAAC, which is in line with the report on the HKU-39849 strain.¹⁵ Our observations revealed that this Leader sequence was identical and present in the different genes of SARS-CoV. Our data further indicated that the sequence between the identical Leader sequence and ATG in different transcripts was variable. The S gene has no space between the common Leader sequence and ATG, and the M gene has the longest spacer sequence. This implied that the common Leader sequence had different merging sites to the sequence of different genes in SARS-CoV. Importantly, we observed that this Leader sequence was identical in different strains of SARS-CoV identified to date (Table 1), while the other sequences of SARS-CoV showed variations.^{11,12,14,44,45}

To evaluate the efficiency of the siRNA, we used the GFP and RFP reporter genes fused with the Leader sequence of SARS-CoV. We proved that the mRNA fused with the Leader sequence did not affect the translation of the GFP and RFP proteins in the cultured cells. As expected, our data showed that the siRNA could block the expression of the reporter genes with the Leader sequence, while it had no effect on those without the Leader sequence. Consistent results were obtained by direct observation of the GFP and RFP proteins using fluorescence microscopy and Western blot analysis. This simple observation model can be used to show the effects of siRNA on the transcription of genes with Leader sequence in other laboratories.

Previously, we reported the use of siRNA targeting the S gene of SARS-CoV to inhibit virus replication.³¹ The probability of gene variation in the S gene might cause randomly selected targeting sequence changes, which would reduce the effectiveness of the designed siRNA. While the Leader sequence of the coronavirus remained identical, we postulated generating siRNA targeting the Leader sequence of SARS-CoV, which is necessary for the transcriptions of various genes of the virus. We considered that this targeting site was more powerful than targeting individual genes and would overcome the various mutations of the other genes in SARS-CoV.

In theory, completely knocking out one of the genes should lead to the blockade of virus replication. However, empirically, siRNA could only knock down the expression of the targeted gene expression. Therefore, designing an siRNA targeting several genes should provide better inhibition of virus replication. To this aim, the Leader sequence in SARS-CoV provided a very useful target because it is part of the mRNAs of each gene critical to SARS-CoV replication. Indeed, our experiments showed that siRNA targeting the Leader sequence was more effective than siRNA for the S gene. Also, our data indicated that the siRNA targeting the Leader sequence was powerful compared to antisense oligodeoxynucleotides. There might be several reasons for the lower effectiveness of the antisense oligodeoxynucleotides including lower transfection efficiency, di-

gestion by DNase and lower transcription of antisense mRNA in the nucleus.

In conclusion, we reported that siRNA targeting the Leader sequence of SARS-CoV could effectively inhibit gene expression and replication of the virus in cultured cells. This work provided a base for gene therapy of SARS. Whether an adenovirus delivery system could be used for the therapy of SARS in animal model and human needs to be further investigated.

Materials and methods

SARS-CoV Leader sequence identification

The sequence of SARS-CoV BJ01 clone (GenBank accession number AY278488) was used to predict the Leader sequence based on the Leader sequence similarity in the coronavirus family. Based on the information of prediction, primers were designed to amplify the cDNA of the transcripts of different genes of SARS-CoV by RT-PCR and the products were sequenced. The primers used were: forward (located upstream of common Leader sequence of SARS-CoV): CCAGGAAAAGCCAACCAAC; reverse (located on the different genes of SARS-CoV): S gene – CCATGCATAGACAGAAGGGAA; M gene – TTAGTGTACTAGCAAAGCAA; E gene – TTAGACCAGAAGATCAGGA; N gene – TGCCTGAGTTGAATCAGC; N gene 3' common genome sequence – GCTATTAATAATCACATGGGA.

The 5'-end of the S gene RNA transcript was identified by using an end-switching 5'-RACE commercial kit (SMART RACE cDNA Amplification kit). Total RNA (1 µg) extracted from virus-infected Vero E6 cells was used for the 5'-RACE reaction in accordance with the manufacturer's instructions. Specific primer (5'-GCTGACGTGCCCAAGTGTCTTGAG-3') located near the 5'-end of the possible S gene was used for PCR and primers (forward: 5'-CCAGGAAAAGCCAACCAAC-3'; reverse: 5'-TAGAAACAGCAAAGAAAGGG-3') were used for nested PCR.

Selection of siRNA targeting sequence

To locate the target site of siRNA in the SARS-CoV leader sequence, we selected those sequences of 22 bp with the structure of AGN₁₈TT. As an empirical design,^{17,33} we chose the sites with GC/AT ratios between 30 and 60%. In matching for those criteria, a uniquely specific target sequence CCAACCAACCTCGATCTC was determined.

This sequence was further confirmed for the specificity by Blasting against Nr and human Genome databases. The synthesized oligos were: forward – 5'-CCAACCAACCTCGATCTCTTCAAGAGAGATCGAGGTTGGTTGGCTTTTGG-3'; reverse – 5'-AATTCAAAAAGCCAACCAACCTCGATCTCTTGAAGAGATCGAGTTGGTTGG-3'.

Vectors, vector construction and antisense RNA synthesis

pBS/U6, a backbone vector with a U6 promoter, kindly provided by Dr Yang Shi, was used for the construction of the siRNA vector. The hairpin cDNA was generated through annealing of the complementary oligos synthesized above and directly inserted into the pBS/U6 vector

through *Apa*I and *Eco*RI sites. The correct clones were verified by *Kpn*I and *Xho*I cutting. Finally, the clones were confirmed by sequencing. This vector was named pBS/U6/L-RNAi.

For the construction of the reporter vectors with a SARS-CoV Leader sequence, pEFBos/GFP or pDsRed1.1/RFP vector was used as backbone and subcloned through *Kpn*I and *Bam*HI sites by inserting the SARS-CoV Leader sequence cDNA, which was generated through annealing of the complementary oligos synthesized as sense: 5'-CCCAGGAAAAGCCAACCAACCTCGATCTCTTGATAGATCTGTTCTCTAACGAAC-3' and antisense: 5'-GATCCGTTCCGTTTAGA GAACAGATCTACAAGAGATCG AGGTTGGTTGGCTTTCTGGGGTAC-3'. The correct clones were verified by *Kpn*I and *Bam*HI cutting, which generated a 60 bp insert. The clones were sequence confirmed. The two reporter vectors with the Leader sequence of SARS-CoV were named pEFBos/L-GFP and pDsRed1.1/L-RFP, where L stands for Leader sequence. The correct expression of the mRNA by the construct was examined by RT-PCR using the primers as shown in Figure 2a: a, SARS-CoV leader sequence forward primer, CCAGGAAAAGCCAACCAA; b, GFP forward primer, ATGGTGAGCAAGGGC GA; c, GFP reverse primer, TTAGTGTACAGCTCGTCCATG; d, RFP forward primer, ATGGTGCGCTCCTCCAA; and e, RFP reverse primer, CTACAGGA CAGGTGGTGG.

The other vectors used in this paper included, pBS/U6/S-RNAi, constructed by our lab;³¹ and pBS/U6/GFP-RNAi, kindly provided by Dr Yang Shi. For the establishment of the stable cell lines based on Vero E6 cells, pcDNA3 was used as backbone to subclone the insert, through *Spe*I and *Eco*RI sites, from the pBS/U6, pBS/U6/GFP-RNAi or pBS/U6/L-RNAi vector with the U6 promoter with GFP-RNAi or L-RNAi sequence. The three new constructs were named pcDNA3/U6, pcDNA3/U6/GFP-RNAi and pcDNA3/U6/L-RNAi.

The antisense oligodeoxynucleotides targeting the different regions of SARS-CoV Leader sequence were directly synthesized and thio-modified. The sequences used are: A1 (5'-GGTTGGTTGGCTTTTCTGG-3'), A2 (for 5'-AGAGATCGAGGTTGGTTGGC-3') and A3 (5'-GATCTACAAGAGATCGAGGT-3'). Three wells of repeats were used in each experiment.

Cell culture and transfection

293T and Vero E6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. 293T cells were plated into six-well dishes at a density of 4×10^5 cells/well and cultured overnight before transfection. A total of 2.5 µg DNA/well was used for the transfection with the CalPhos[®] (ClonTech) transfection reagent according to the manufacturer's protocol. In the case of large-scale transfection, 293T or VERO E6 cells was plated in 60 mm dishes at a density of 11×10^5 cells/dish and a total of 6 µg DNA/well were used for transfection with CalPhos[®] (ClonTech). The transfected cells were cultured for at least 48 h or longer for further experiments. The antisense oligodeoxynucleotides were transfected in 24-well plates at a density of 1×10^5 cells/well and a total of 1 µg DNA/well with Lipofectamine 2000.

Establishment of stable cell line

The construct pcDNA3/U6/L-RNAi, pcDNA3/U6 or pcDNA3/U6/GFP-RNAi was transfected into Vero E6 cells and selected with G418 (1.5 mg/ml) for 4 weeks. In all, 20 single-cell clones for each of the transfected constructs were selected and amplified. The correct clones were examined by PCR using the genomic DNA of cloned cells. The PCR primers used were: forward, 5'-CCTGCCCCGGTTAATTTG-3'; reverse, U6, 5'-AGAAC TAGTGGATCCCCCGG-3'. In the final experiment of SARS infection, at least three clones were mixed as a pool to represent the clone of the recombinant fragment of U6, U6/GFP-RNAi or U6/L-RNAi, which was also the name of the cell lines established.

DNA, RNA isolation and PCR, RT-PCR analysis

Genomic DNA or total RNA from the cells was extracted using Trizol (Invitrogen), respectively, with the addition of RNAase or DNAase. The PCR reaction was performed using the primers indicated in each experiment. The PCR reaction conditions are described in the figure legends. RT-PCR was carried out using One Step RNA RT-PCR kit (Takara, Japan) with 1 µg of the total RNA for each sample, according to the manufacturer's protocol.

SARS-CoV culture and infection

SARS-CoV (strain, BJ01, GenBank accession number AY278488), provided by the Beijing Institute of Microbiology and Epidemiology, AIMMS, China, was propagated on Vero E6 cells. The virus was released from the infected cells by three cycles of freezing and thawing. For the test of inhibition of the virus by siRNA, 3×10^5 cells (U6, U6/GFP-RNAi or U6/L-RNAi stable cell line) in 24-well plates were infected with 5×10^5 PFU of SARS-CoV (BJ01), in a final volume of 0.2 ml of DMEM with 2% FBS for 2 h at 37°C. Cells were then washed once with phosphate-buffered saline (PBS; pH 7.4) added, 0.5 ml complete media was added to allow growth for 72 h at 37°C with 5% CO₂. For different SARS-CoV infection experiments, the same ratio of cell/virus was used. All those operations were performed in a bio-safety P3 level lab.

Virus titration

The Vero E6 cells in 96-well plates were challenged by a series of dilutions of sample virus. The virus was allowed to infect the cells for 2 h and then was completely withdrawn by washing with PBS. After incubation for 72 h, the cells were stained and the dilution of the most infected cells (with no staining) was regarded as the titer of the virus (PFU).

Western blot analysis

For the detection of the GFP protein fused with a SARS-CoV Leader sequence, cells were harvested 48 h post-transfection and lysed with 0.2 ml of cold lysis buffer (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM Na₂VO₃, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol-phosphate, 1 mM PMSF, 5 µg/ml aprotinin and 5 µg/ml leupeptin, pH 7.5). In all, 10 µg of total cell lysis protein per sample was separated on 12% SDS-PAGE, electrotransferred to nitrocellulose membrane (Hybone ECI, Amersham Pharmacia Biotech) at 100 V for 1 h, followed by Western blot analysis.

Antibodies directly to GFP and β-actin were obtained from Santa Cruz, CA, USA. The signals were detected by an enhanced chemiluminescence (ECL, Amersham).

Northern blot

Total RNA (10 µg) from different cells infected by SARS-CoV was subjected to a 1.3% formaldehyde-agarose gel and transferred to a nitrocellulose membrane. The probe from SARS-CoV were labeled with ³²P using a Prime-a-Gene kit (Promega), according to the manufacturer's protocol. Hybridization was performed at 68°C for 2 h in Hybridization Butter (Promega). Membrane was washed in 2 × SSC with 0.1% SDS at 50°C three times and autoradiographed by PhosphorImager (DNA Storm, Amersham Pharmacia Biotech). The probe used was generated by digestion of N gene cDNA using *EcoRI*, which corresponds to the 3'-end of SARS genome (BJ01, AY278488, 29458–29692).

Cell staining and fluorescence microscopy observation

At 2 days after transfection with the reporter constructs, cells were harvested and analyzed with the aid of a fluorescence microscope (Leica, Deerfield, IL, USA) using objective × 20, and the data were acquired with a Sony digital charge-coupled device camera and processed by Adobe PHOTOSHOP software. The cells infected with SARS-CoV were stained for 30 min with 50 µl of violet staining solution (50 mg crystal violet in 20 ml ethanol plus 100 ml dH₂O) and washed for 5 min with 100 µl of destaining solution (50% ethanol, 0.1% acetic acid in dH₂O). The cell morphology was observed using a microscopy. For the experiments of reporter gene expression, cells were directly observed by Nikon TE300 fluorescence microscope. Pictures were taken with a CCD camera (SPOT, Diagnostic Instrument Inc., USA) equipped with a computer-based, image acquisition system.

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