

Identification of Effective siRNA Blocking the Expression of SARS Viral Envelope E and RDRP Genes

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Abstract

A cell-based assay was developed to screen small interference RNA (siRNA) to block the expression of two genes of the severe acute respiratory syndrome (SARS) virus. These two genes encode RNA-dependent RNA polymerase (RDRP) and envelope E protein. The RDRP plays an essential role in viral RNA replication where envelope E protein is involved in envelope formation and virus assembly. The RDRP and envelope E genes, based on published sequences, have been synthesized and cloned into mammalian expression vectors. In addition, four siRNA sites for the RDRP gene and two siRNA sites for envelope E gene were designed and tested. The siRNA or short hairpin RNA (shRNA) expression cassettes were co-transfected with the SARS viral RDRP or envelope E expression vectors into NIH 3T3 cells. The expression levels of RDRP and envelope E genes were examined by reverse transcription followed by quantitative real-time polymerase chain reaction (PCR). Two of the siRNA expression cassettes for RDRP successfully inhibited the expression of the gene, whereas both of the siRNA expression cassettes for envelope E decreased approx 90% of the envelope E gene expression. The siRNA and shRNA for one of the siRNA sites of the RDRP gene were also tested, and it was found that both inhibited exogenous RDRP expression in a dose-dependent manner. These siRNA molecules could be used to examine the function of these genes in SARS virus replication and assembly. Furthermore, these molecules could potentially be developed into therapeutic agents for the treatment of patients with SARS.

Index Entries: RNAi; SARS; RNA-dependent RNA polymerase (RDRP); envelope E.

1. Introduction

Regulation of gene expression by small interference RNA (siRNA) has been used as a powerful tool to investigate gene function (*1–6*). In addition siRNAs that could effectively inhibit certain gene expressions can be developed potentially as therapeutic drugs for the treatment of human diseases (*7–9*). Many technologies have been developed to inhibit gene expression using RNA interference (RNAi), including polymerase chain reaction (PCR)-based approaches (*10,11*). For example, sense and antisense strands of

siRNA could be generated under the control of specific promoters to form double-stranded siRNA in mammalian cells. Another possible way to produce siRNA is through a hairpin structure; in this case, the siRNAs are expressed as hairpin structures and then processed to siRNA intracellularly. In this report, both approaches have been used to test a number of siRNA molecules focusing on severe acute respiratory syndrome (SARS) envelope E protein and RDRP.

The etiologic agent for SARS is a new type of coronavirus (*12*). The whole genome of this RNA virus is 29,727 nucleotides in length with 11 open

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reading frames (13). Detailed sequencing analysis of the viral genome shows that several genes play an essential role in viral replication and formation. These genes, including RDRP, N protein, and envelope E protein, could be therapeutic targets for antiviral drug development (14). Inhibition of the expression or activity of RDRP could block replication of the viral genome. On the other hand, N protein and envelope E protein are important structural proteins and inhibition of their expression may therefore interfere with the structure of nucleocapsid, and arrest envelope formation and virus assembly. The siRNAs for these genes have been designed and screened. Results obtained demonstrate that a number of siRNA molecules could effectively inhibit the expression of RDRP and envelope E genes in transfected NIH 3T3 cells. These siRNA molecules could act as a useful tool for the study of SARS virus replication and assembly. Furthermore, these siRNAs could be developed into antiviral drugs for the treatment of patients with SARS.

2. Materials and Methods

2.1. Plasmid Construction

The SARS envelope E gene was synthesized *de novo* in the laboratory (15) based on the published sequence (GenBank accession no. AY451891). Primers used for the PCR reaction were 5'-CTT ATG TAC TCA TTC GTT TCG GAA G-3' and 5'-ATAGTT AGT TCG TTT AGA CCA GAA G-3'. The PCR product was cloned into mammalian expression vector pCDNA3.1 (named envE-pCDNA3.1) and used for NIH 3T3 cell transfection. The SARS RNA-dependent RNA polymerase (*RDRP*) gene was synthesized (Shanghai GeneBase Gene-Tech Co., Ltd., China) based on the *RDRP* sequence (GenBank accession number AY268070.1). Initiation codon ATG and stop codons TGA TAA were added to the synthesized *RDRP* gene. The *RDRP* gene was PCR amplified using primers 5'-TTG CCT ACG CAG ACT GTT GAT TC-3' and 5'-CCC ACA GCA TCT CTA GTT GCA TG-3'. The PCR product was cloned into expression vector pCDNA3.1 (named *RDRP*-pCDNA3.1) and used for transfection.

2.2. Construction of Expression Cassettes for Sense and Antisense siRNA

The siRNA expression cassettes were generated using LineSilence™ RNAi Transcription Kit (Allele Biotechnology & Pharmaceuticals, Inc.). A U6 RNA-based polymerase III promoter and a modified terminator were used in the cassettes to facilitate high-level siRNA expression. (see Fig. 1 for the whole scheme). The following RNAi targets were selected: Ei1: AAA CCA ACG GTT TAC GTC TAC; Ei2: AAG GAG TTC CTG ATC TTC TGG T; Ri1: AAT GTC AAC CGC TTC AAT GTG GC; Ri2: AAA TAC CAC GTC GCA ATG TGG C; Ri3: AAG GAC ATG ACC TAC CGT AGA C; Ri4: AAG CTA TTC GTC ACG TTC GTG C. A nonsense fragment with a sequence of 5'-AAG TTC ACC TTG ATG CCG TTC-3' (named NS) was also used in the experiments. The sequences were examined using BLAST program and no homologies were found in human or other mammalian genomes.

The PCR reactions were carried out to generate sense and antisense siRNA driven by a U6 promoter. The upstream primer starting from the U6 promoter was supplied with the kit and used for all PCR reactions. The downstream primers were synthesized and listed in Table 1 (Shanghai Genebase Gene-Tech Co., Ltd.). PCR reactions were performed in a total volume of 50 μ L with a 1-ng template, supplied in the LineSilence RNAi Transcription Kit, 0.2 mM deoxynucleotide 5'-triphosphate (dNTP), 0.6 μ M of both upstream and downstream primers, 5 μ L of the 10X polymerase reaction buffer, and 5 U of *exTaq* polymerases (Takara, Dalian, China). The PCR reactions were initiated at 94°C for 5 min to denature the DNA template. In subsequent cycles, denature of DNA template was carried out at 94°C for 30 s with annealing and extension being performed in a single step at 72°C for 90 s. This reaction was repeated for 38 times. Finally, the reaction was incubated at 72°C for 10 min. The PCR products were then isolated by extraction from 1% agarose gel and purified with a PCR clean-up kit (V-gene Biotech Ltd.). The purified PCR products were used for transfection.

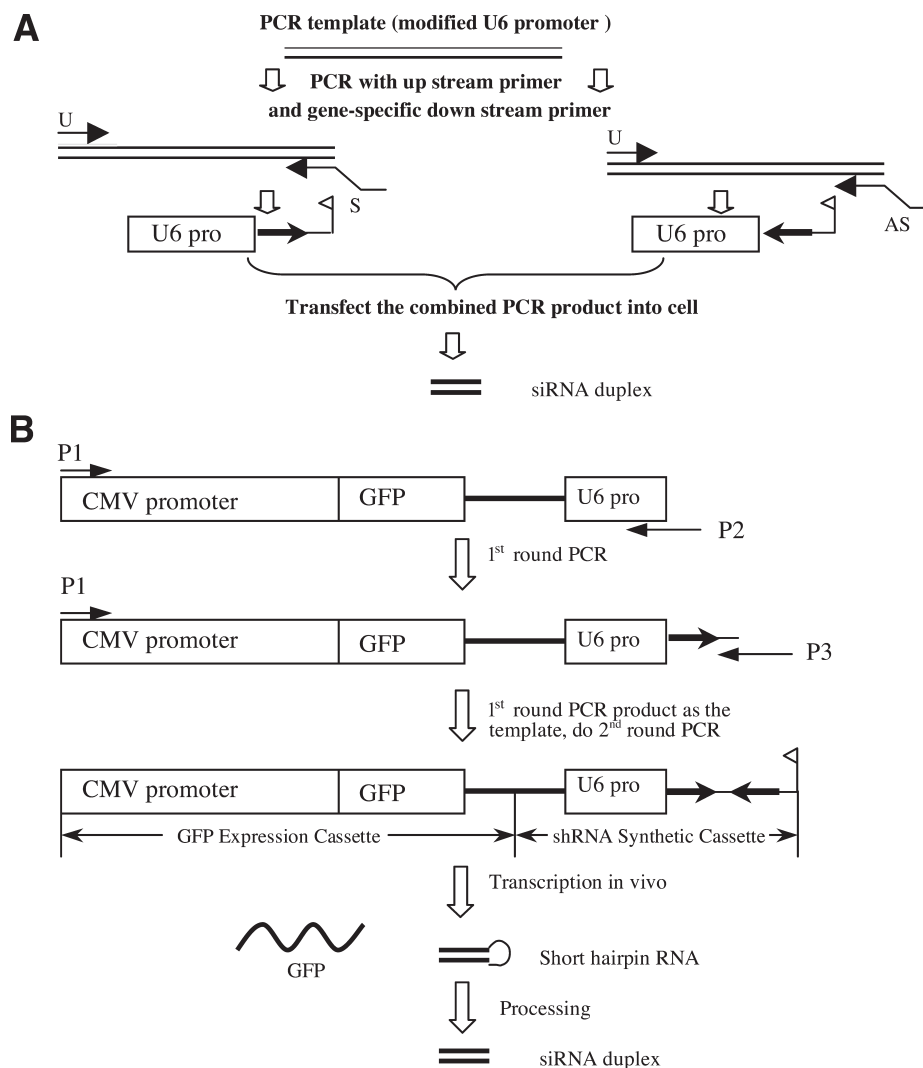


Fig. 1. Generation of siRNAs and shRNA. (A) Expression cassette for sense and antisense siRNA. (B) Expression cassette for short hairpin RNA (shRNA) that is subsequently processed to siRNA intracellularly.

2.3. Construction of Expression Cassettes for shRNA

The Ri3 site from the *RDRP* gene was selected for the generation of short hairpin RNA (shRNA) expression cassette. **Figure 1B** shows the procedure for the production of shRNA. The primers used for PCR reaction were: shRNAi-up: GAA GGT ACC TCG AGC TGG TTC TTT CCG CCT CAG AAG, shRi-1: 5'-CCA AGC TTC GTC TAC GGT AGG TCA TGT CCG GTG TTT CGT CCT TTC CAC AA-3' and shRi-2: 5'-GGA CTC

GAG AAA GGA AAA AGG ACA TGA CCT ACC GTA GAC CAA GCT TCG TCT-3'. Two rounds of PCR reactions were carried out to generate the shRNA expression cassette. For the first round, the PCR reaction was then preformed in a total volume of 50 μ L with a 1-ng template supplied by the Nitto Denko Technical Corporation (NDT) siRNA Cassette kit, 0.2 mM (dNTP), 0.6 μ M of both upstream primer shRNAi-up and downstream primers shRDRPi, 5 μ L of 10X polymerase reaction buffer and 2.5 U *exTaq* polymerases

Table 1
Primers for Generating siRNA Targeting to SARS Viral Envelope E (Set A) and RDRP (Set B)

Primer	Sequence of primers
Set A	
Ei1-1	5'-caaaaac tgtaaa AAACCAACGGTTTACGTCTAC gg tgt ttc gtc ctt tcc aca aga-3'
Ei1-2	5'-caaaaac tgtaaa GTAGACGTAAACCGTTGGTTT gg tgt ttc gtc ctt tcc aca aga-3'
Ei2-1	5'-caaaaac tgtaaa AAGGAGTTCCTGATCTTCTGGT gg tgt ttc gtc ctt tcc aca aga-3''
Ei2-2	5'-caaaaac tgtaaa ACCAGAAGATCAGGAACCTCCTT gg tgt ttc gtc ctt tcc aca aga-3'
Set B	
Ri1-1	5'-caaaaac tgtaaa AATGTCAACCGCTTCAATGTGGCgg tgt ttc gtc ctt tcc aca aga-3'
Ri1-2	5'-caaaaac tgtaaa AAGCCACATTGAAGCGTTGACA gg tgt ttc gtc ctt tcc aca aga-3'
Ri2-1	5'-caaaaac tgtaaa AAATACCACGTCGCAATGTGGC gg tgt ttc gtc ctt tcc aca aga-3'
Ri2-2	5'-caaaaac tgtaaa AAGCCACATTGCGACGTGGTAT gg tgt ttc gtc ctt tcc aca aga-3'
Ri3-1	5'-caaaaac tgtaaa AAGGACATGACCTACCGTAGACgg tgt ttc gtc ctt tcc aca aga-3'
Ri3-2	5'-caaaaac tgtaaa AAGTCTACGGTAGGTCATGTCCgg tgt ttc gtc ctt tcc aca aga-3'
Ri4-1	5'-caaaaac tgtaaa AAGCTATTCGTCACGTTCTCGTGC gg tgt ttc gtc ctt tcc aca aga-3'
Ri4-2	5'-caaaaac tgtaaa AAGCACGAACGTGACGAATAGCgg tgt ttc gtc ctt tcc aca aga-3'

(Takara, Dalian, China). The PCR reaction was initiated at 94°C for 5 min to denature the DNA template. In subsequent cycles, denaturation was carried out at 94°C for 30 s, annealing at 66°C for 30 s, and extension at 72°C for 150 s. This reaction was repeated for 38 cycles. Finally, the reaction was incubated at 72°C for 10 min. The PCR products were isolated by extraction from 1% agarose gel and purified with a PCR clean-up kit (V-gene Biotech Ltd.). For the second round PCR, 1 to 10 ng of purified PCR products were used as the template to carry out PCR using the same conditions as the first round of PCR. The final PCR products were named shRi and used for transfection.

2.4. Cell Culture and Transfection

The National Institutes of Health (NIH) 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C. Transfection was performed using FuGENE6 (Roche) in a 24-well plate according to the instructions from the manufacturer. Equal amounts of sense and antisense siRNA expression cassettes were mixed in advance and 0.2 µg of the mixture and 0.1 µg of envE-pcDNA3.1/RDRP-pcDNA3.1 were used for transfection in NIH 3T3

cells. For the dose-dependent RNAi experiments, 0.1 µg of RDRP-pcDNA3.1 plasmid was co-transfected with either 0, 0.04 µg, 0.12 µg, and 0.2 µg of Ri3 fragments or with 0, 0.02, 0.06, and 0.1 µg of shRi fragment. Total amount of transfected DNA was kept constant at 0.3 µg/well by adding NS control to the DNA mixture.

2.5. RNA Isolation and Quantitative RT-PCR

Forty-eight hours after transfection, total RNA was isolated using Trizol (GIBCO). RNA was first treated with DNase I to eliminate DNA contamination. The first-strand cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen). One microgram of total RNA was used and the reaction was performed at 37°C for 1 h according to the instructions from the manufacturer and then the cDNA was diluted 50 times for the subsequent quantitative PCR. Quantitative PCR reaction was carried out using the DNA Engine Opticon 2 System (MJ Inc.). The PCR reactions were then performed in a total volume of 20 µL with 1 µL of cDNA, 0.2 mM dNTP, 0.4 µM of both upstream and downstream primers, 2 µL of the 10X polymerase reaction buffer, 1 U of *Taq* polymerase (BioAsia, Sanghai), and 0.2 µL of

SYBR green I (Ope-Tech Co., Ltd., Shanghai). The reactions were initiated at 95°C for 5 min to facilitate denaturation of the template. In subsequent cycles, denaturation was carried out at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s, with a plate read at 82°C for 1 s. The PCR reactions and plate reads were repeated for 40 cycles. Each reaction was performed in triplicate. The C_t values provided from the real-time PCR instrumentation were imported into SigmaStat software, and the relative changes in envelope E and RDRP gene expression were analyzed with the $2^{-\Delta C_t}$ method (16) using β -actin as the internal control. The relative changes were averaged and expressed as mean \pm SEM (standard error of mean). The statistical comparisons between groups of control and siRNA/shRNA-treated NIH 3T3 cells were analyzed using the Student's *t*-test, and for dose-dependent experiments, the statistical comparisons among all the groups were analyzed using one-way analysis of variance (ANOVA) test.

3. Results

We have developed a cell-based assay to screen and identify siRNA to block the expression of SARS virus genes. Envelope E and RDRP genes were synthesized and transfected in NIH 3T3 cells. A real-time PCR experiment showed that both envelope E and RDRP RNA were expressed in the cells after transfection (data not shown). PCR-based RNAi technology was applied to knockdown the expression of these two viral genes. Two siRNA molecules for envelope E gene and four siRNA-targeting RDRP genes were designed and used. The RNAi targets were selected based on published strategies (17) and on-line siRNA design tools (Ambion siRNA Target Finder). First, the AAGN17C sites from 70 to 100 nucleotides downstream of the initiation codon was searched initially. It is important to avoid 5' or 3' untranslated regions as these sequences may contain regulatory protein-binding sites. The target sequences had approx 30–50% of G and C. It is also essential to avoid 4–5 continuous stretch of “A” and “T” as it may act as internal terminator. Finally, the Blast program for each selected site

was carried out to ensure the specificity. The siRNAs for envelope E gene used in the experiment were located at positions 157 to 177 (Ei1) and 206 to 227 (Ei2). The PCR products of the siRNA were transfected in NIH 3T3 cells expressing the envelope E gene. Forty-eight hours after transfection, the envelope E RNA was measured using quantitative real-time PCR. The result showed that the expression of envelope E gene was downregulated with statistical significance after both siRNA treatments. **Figure 2A** shows Ei1 siRNA downregulated 89% of the gene expression (Student's *t*-test, $p < 0.05$), and Ei2 siRNA inhibited approx 97% (student's *t*-test, $p < 0.01$).

Furthermore, four siRNAs for RDRP were designed based on positions sequentially located at 118 to 140 (Ri1), 224 to 245 (Ri2), 394 to 415 (Ri3), and 488 to 509 (Ri4). The results demonstrated that Ri1 and Ri3 almost completely blocked the expression of the RDRP gene (Student's *t*-test, $p < 0.01$), Ri2 inhibited approx 60% (Student's *t*-test, $p < 0.01$), and Ri4 had no effect (**Fig. 2B**). The experiment was repeated three time using Ri1 and Ri3 and the results were reproducible (data not shown). It was further demonstrated that the RNAi effects were dose-dependent using Ri3 as an example (**Fig. 3**). In addition, it was also shown that the shRNA expression cassette targeting the Ri3 site of SARS RDRP possessed the same significant RNAi effect in a similar dose-dependent manner (**Fig. 3**).

4. Discussion

It has been shown that siRNAs were interactive with various protein factors to form the RNA-induced silencing complex (RISC) (18). The RISC was activated and guided by the antisense strand of siRNA and recognized the complementary RNA. The RISC and complementary RNA complex recruited endoribonucleases. Subsequently, the complementary RNA was cleaved by the endoribonucleases. The recognition may be biased to the strand selection during siRNA–RISC assembly and contribute to the final RNAi efficiency (19,20). Based on statistical analysis, the effective siRNA had A/T at the 5' end of the

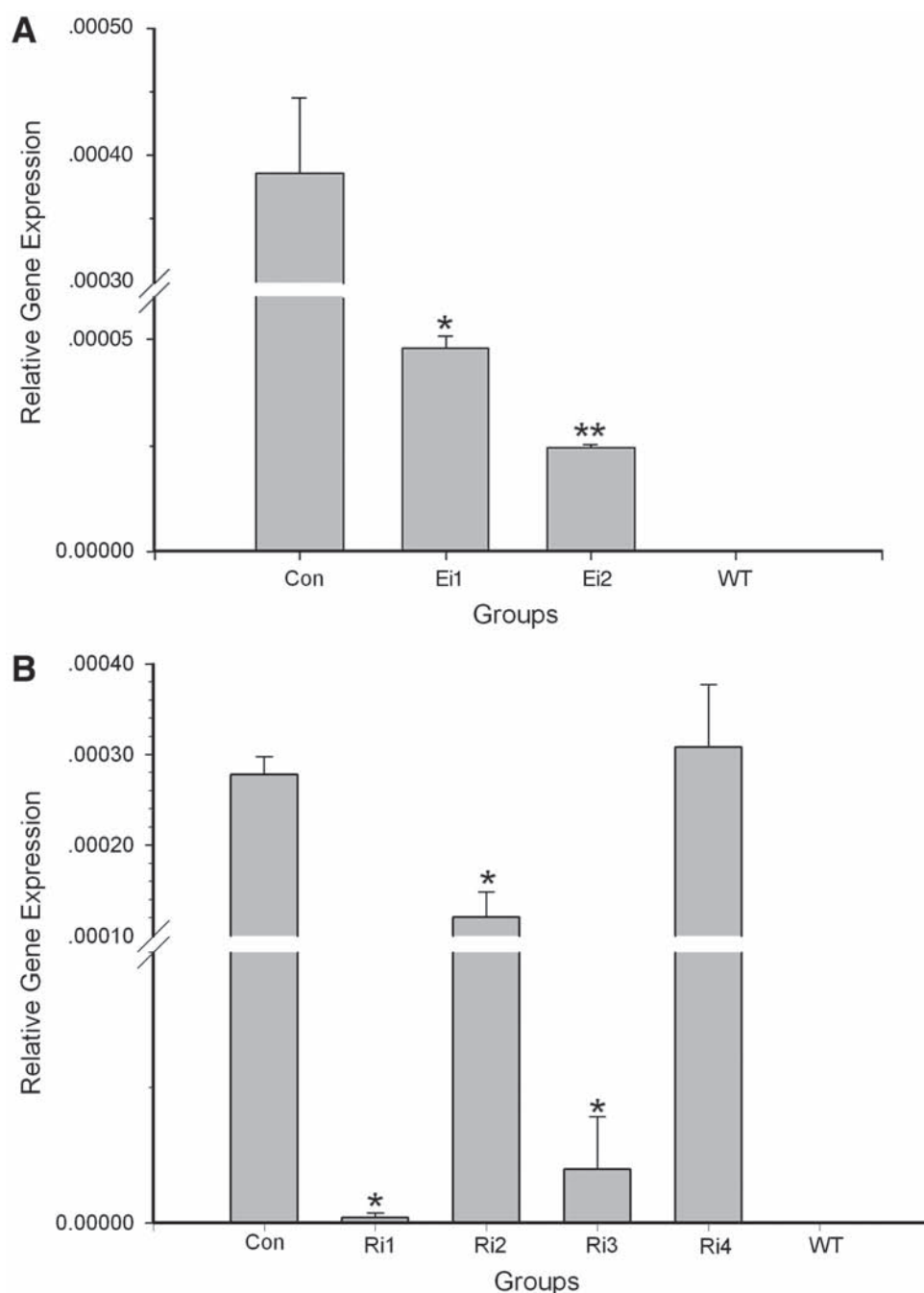


Fig. 2. Relative expression of SARS envelope E gene and *RDRP* gene measured by quantitative real-time PCR in NIH 3T3 cells. The $2^{-\Delta C_t}$ method was used to analyze envelope E gene and *RDRP* gene expression. (A) NIH 3T3 cells were co-transfected with envelope E gene and RNAi targets at Ei1 and Ei2 sites. (B) NIH 3T3 cells were co-transfected with *RDRP* gene and RNAi targets at Ri1, Ri2, Ri3, and Ri4 sites. Con: NIH 3T3 cells transfected with envelope E/pcDNA3.1 or *RDRP*/pcDNA3.1; WT: nontransfected NIH 3T3 cells. The columns present the mean of the relative gene expression with the beta-actin as the reference gene. The bar presents the standard errors of the mean (SEM) of the relative gene expression. Student's *t*-test; * $p < 0.05$, ** $p < 0.01$ vs control.

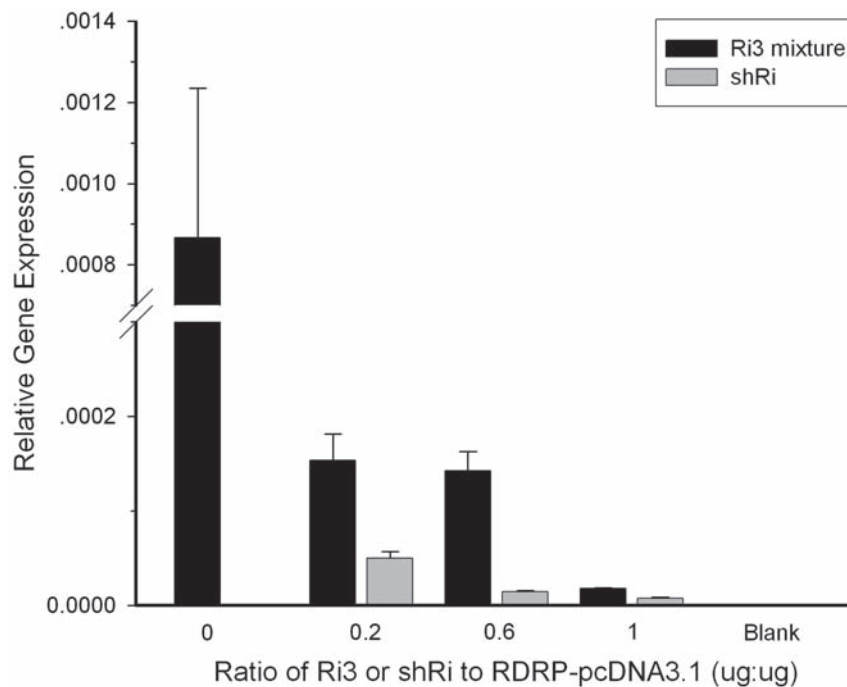


Fig. 3. Dose-dependent RNAi effect on SARS *RDRP* gene using siRNA expression cassettes or shRNA expression cassettes. RDRP-pcDNA3.1 was co-transfected with either Ri3 or shRNA into NIH 3T3 cells. The ratio of Ri3 and shRNA to RDRP-pcDNA3.1 were shown. A nontransfection NIH 3T3 sample was included as a blank control. The relative expression levels of *RDRP* gene were measured by quantitative real-time PCR, and $2^{-\Delta Ct}$ method was used. The columns present the mean of the relative gene expression, with the beta-actin as the reference gene. Black columns represent the relative gene expression treated with Ri3 siRNA expression cassettes; gray columns show the relative gene expression treated with shRNA expression cassette. The bars present the standard errors of the mean (SEM) of the relative gene expression. One-way ANOVA, $p = 0.002$.

antisense strand and G/C at the 5' end of the sense strand. In addition, the sequence should have high A/T content in the 5' terminal one-third of the antisense strand without any GC stretch for more than nine nucleotides in length (18). In the current experiment, Ri1 and Ri3 siRNAs may have higher affinity for protein factors and form stable RISC. It is also possible that these siRNAs were more stable and performed strong catalytic activity in the cell. This may lead to an explanation for the different inhibition efficiency of these four siRNAs of the *RDRP* gene. This could provide important information to design and identify highly effective siRNA molecules for the SARS genes. Furthermore, it was shown that the PCR-based siRNA expression cassettes as well as the shRNA were effective.

In summary, the RNA-based siRNA expression cassette was applied to screen the effective RNAi sites in SARS envelope E and *RDRP* genes. A number of siRNAs have been identified that effectively blocked the expression of these genes. These siRNA molecules may be useful for further studies on the mechanism of the SARS coronavirus action, and could potentially be developed as therapeutic agents for SARS treatment.

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