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RNAi and SARS

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Abstract

Outbreaks of severe acute respiratory syndrome (SARS), caused by the newly identified coronavirus SARS-CoV, occurred between 2002 and 2003. A number of prophylactic and therapeutic strategies for SARS were reported but specific treatment for this disease has not been established yet. RNA interference (RNAi) is triggered by the presence of a double-stranded RNA (dsRNA), and results in specific degradation of mRNA containing the same sequence. Recently it has been shown that siRNA can inhibit replication of various kinds of viruses including SARS-CoV in vitro and in vivo. This review will first provide current information on SARS-CoV and virus-targeting siRNA, and will then discuss improvement of RNAi technology for efficient and stable silencing.

Introduction

Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) is a novel coronavirus identified in late March 2003, and the WHO announced on April 16 that this coronavirus was the definitive cause of SARS [1-4]. SARS represents typical influenza-like symptoms such as high fever, myalgia, dyspnea, lymphopenia and pneumonia, with the severe acute breathing problems which raise the overall mortality to 10%. A total of 8098 cases of SARS were reported from 26 countries, and 774 patients died as a result between 1 November 2002 and 31 July 2003 [5].

The outbreak of SARS has posed a necessity to develop strategies to deal with emerging infectious diseases. Certain empirical measures such as antibiotics, antiviral agents, corticosteroids, and interferons were applied to treat patients suffering from SARS [6-9], but no specific and effective strategy has not been established yet. Researchers have made efforts to find new therapeutic strategies and tested many kinds of treatments including RNA-mediated interference (RNAi) [10-19].

RNAi was originally discovered in plants and then found in other organisms such as *Caenorhabditis elegans*, *Drosophila*, and vertebrates [20-22]. It is an evolutionarily conserved process for suppression of gene expression [21, 23-25]. In this process, recognition of double-stranded RNA leads to the production of small interfering RNAs (siRNAs) of 21 to 22 nucleotides (nt), which associate with a multiprotein complex known as the RNA-induced silencing complex and ultimately target homologous mRNA for degradation based on complementary base pairing.

RNAi has been successfully used in blocking the replication of some viruses such as human immunodeficiency virus and human hepatitis C virus [26-30]. Since SARS-CoV is an RNA virus, RNAi could be a reasonable approach for therapeutic purposes against SARS.

In this chapter, the current information on SARS-CoV and RNAi will be reviewed and RNAi as a therapeutic strategy against SARS will be discussed.

Genome organization of SARS-CoV

Coronaviruses, a genus of the coronaviridae family, are large, enveloped, positive-stranded RNA viruses, and can cause many infectious diseases in humans and animals [31, 32]. Before the SARS outbreak, coronaviruses consisted of three groups. Group I includes human HCoV-229E, porcine transmissible gastroenteritis virus (TGEV), whereas HCoV-OC43, mouse hepatitis virus (MHV) and bovine coronavirus (BCoV) belong to group II. Group III includes avian infectious bronchitis virus (IBV) and turkey coronavirus (TCoV).

Sequence analysis of the entire SARS-CoV RNA genome indicated that the virus genome was very similar to previously characterized coronaviruses, with the same order of the genes (replicase (R), spike (S), envelope (E), membrane (M) and nucleocapsid (N) gene), where there are few accessory genes or motifs spanning between the structural genes and at the 3' UTR (untranslated region) (Fig. 1 and table 1) [2, 33-35].

All coronaviruses have a very similar organization in their functional and structural genes, but the arrangement of the non-essential genes is notably different among the subgroups. Group I coronaviruses are chiefly characterized by the presence of ORFs following the N gene. Group II coronaviruses include two additional ORFs, non-structural protein 2 (ns2) and HE gene, located between ORF 1b and the S gene. Only group III coronaviruses have ORFs between the M and N gene, and a conserved stem-loop motif s2m at their 3' UTR (Fig. 1 and table 1). Accessory ORFs are found between the S and E genes in all of the subgroups.

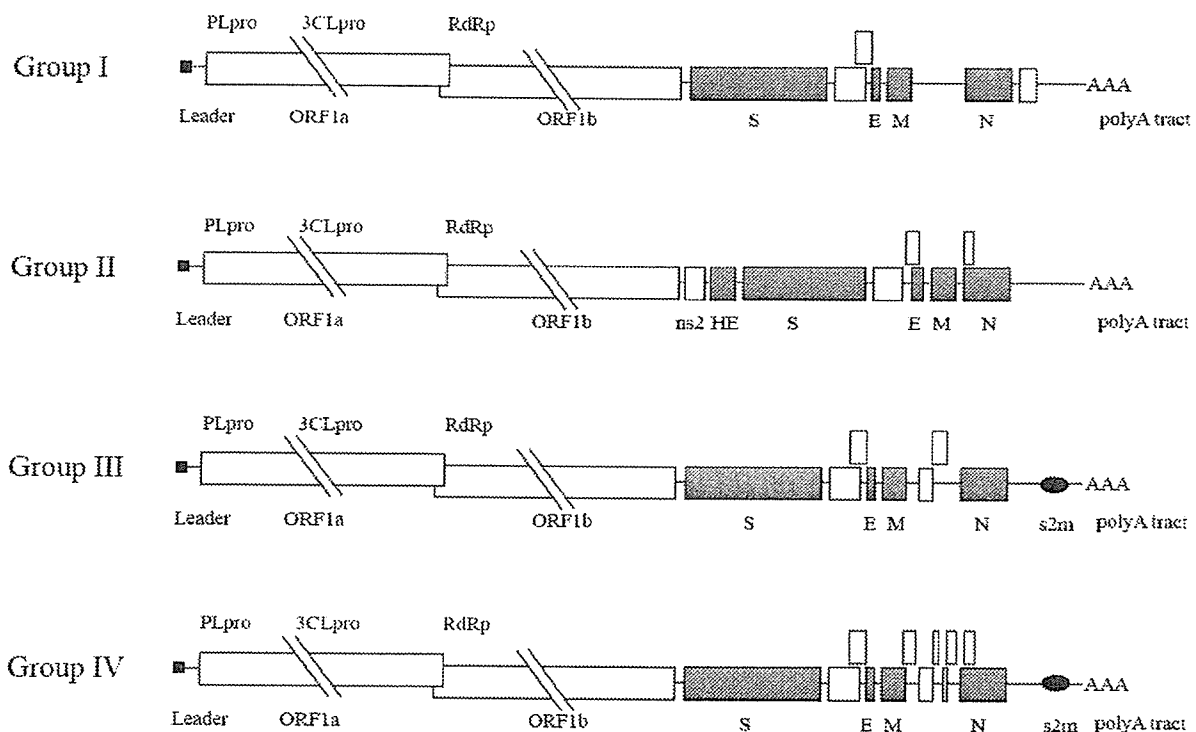


Figure 1. Gene organization of coronaviruses. Coronaviruses are divided into 4 classes on the basis of their genome organization. SARS-CoV represents a new class of coronavirus, group IV. The boxes indicate ORFs and shaded boxes are coding sequences for structural proteins. PLpro: papain-like cysteine protease; 3CLpro: 3C-like cysteine protease; RdRp: RNA-dependent RNA polymerase; HE: Haemagglutinin esterase; S: Spike protein; E: Envelope protein; M: Matrix protein; N: Nucleocapsid protein.

Table 1. Features of SARS-CoV genome sequence and subgenomic transcripts.

mRNA	ORF				Start	End	No. of a.a.	No. of Bases	Frame
	Marra et al.	Rota et al.	Thiel et al.	Zeng et al.					
mRNA1	ORF 1a	ORF 1a	ORF 1a	ORF 1a	265	13398	4382	13149	+1
mRNA1	ORF 1b	ORF 1b	ORF 1b	ORF 1b	13398	21485	2628	7887	+3
mRNA2	S protein	S protein	S protein	S protein	21492	25259	1255	3768	+3
mRNA3	ORF 3	X1	ORF 3a	X1	25268	26092	274	825	+2
mRNA3	ORF 4	X2	ORF 3b	N/R	25689	26153	154	465	+3
mRNA4	E protein	E protein	E protein	N/R	26117	26347	76	231	+2
mRNA5	M protein	M protein	M protein	M protein	26398	27063	221	666	+1
mRNA6	ORF7	X3	ORF6	N/R	27074	27265	63	192	+2
mRNA7	ORF 8	X4	ORF 7a	X2	27273	27641	122	369	+3
mRNA7	ORF 9	N/R	ORF 7b	N/R	27638	27772	44	135	+2
mRNA8	ORF 10	N/R	ORF 8a	X3	27779	27898	39	120	+2
mRNA8	ORF 11	X5	ORF 8b	N/R	27864	28118	84	255	+3
mRNA9	N protein	N protein	N protein	N protein	28120	29388	422	1269	+1
mRNA9	ORF 13	N/R	ORF 9b	N/R	28130	28426	98	297	+2

Pairwise sequence homology search among the accessory ORFs at the S-E intergenic region of the SARS-CoV (ORF3a,b) and all other coronaviruses shows no significant sequence homology [33, 34, 36] but they are homologous within subgroups. The ORF 5a/5b of group III coronaviruses and ORFs 6-8 of the SARS-CoV are located in a homologous region, but they do not show any significant sequence homology.

The phylogenetic analysis by the comparison of the deduced amino acid sequences of the replicase gene and four structural genes (S, E, M, N) with other coronaviruses was performed [33, 34, 36]. The conclusions drawn by the different research groups were similar, with the observation that SARS-CoV itself forms a distinct cluster, the fourth group of Coronaviridae. Moreover, no detectable recombination event was concluded in the similarity plot on the whole genome alignment with other coronaviruses [36]. The divergence of ORFs between the SARS-CoV and other known coronaviruses suggests that the SARS-CoV might have been circulating in other animal hosts long before its emergence and somehow crossed into a human host by a sudden mutation event or a RNA recombination event with unknown sources.

Infection of SARS-CoV and replication of its genome

Virus entry

The entry of coronaviruses involves three steps, namely, attachment, receptor binding and virus-cell fusion, which are mediated by viral envelope proteins (Fig. 2). The S glycoproteins, which form large petal-shaped spikes on the surface of the virion, have a molecular mass of 150-180 kDa and can be divided into three structural domains: a large external N-terminal domain (consisting of subdomains S1 and S2), a transmembrane domain and a short

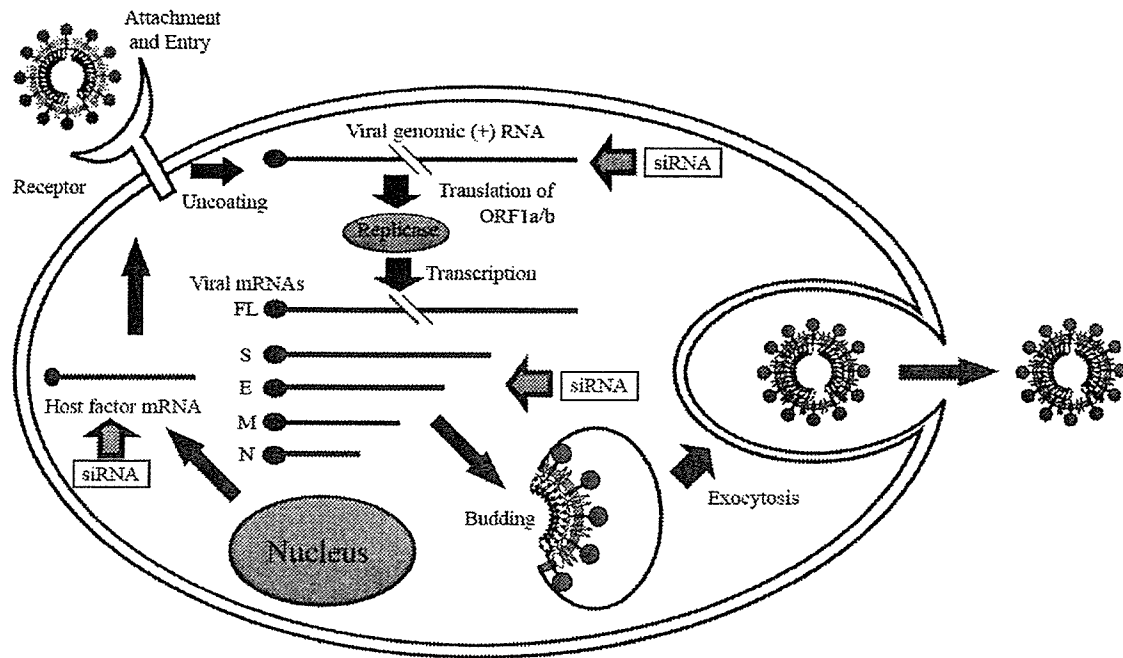


Figure 2. Replication of SARS-CoV and potential targets of siRNA. The life cycle of SARS-CoV starts with the interaction between S protein and the cellular receptor. Silencing the virus receptor may prevent entry of the virus and S protein may also be a good target for RNAi. The RNA genome of the virion is released into the cytoplasm after attachment of the virion to the host receptor, and the virus produces polyproteins encoding ORF1a and 1b by using the translational machinery in host cells. Cleavage by virally encoded proteases yields the components required for assembling the viral replicase complex. These components in replicase complex including RNA-dependent RNA polymerase are potential siRNA targets. In addition to the viral genomic RNA, the subgenomic RNAs encoding E, M and N may be degraded by siRNAs.

C-terminal cytoplasmic domain[37]. The receptor for SARS-CoV is considered to be angiotensin-converting enzyme 2 (ACE2), which is essential in binding of S1 subdomain to the surface of permissive cells[38]. Fine mapping on the N-terminal unit of the spike protein indicates that the receptor-binding domain is probably located between the residues 303 and 537 [39].

Replicase gene

After the step of virus entry, large polyproteins (ORF1a and 1b) are synthesized directly from the capped genomic RNA. The replicase gene of the SARS-CoV includes many kinds of polyproteins which are produced as a consequence of the proteolytic processing of ORF1a and 1b [34]. The expression of ORF1b involves -1 ribosomal frameshifting, a process that essentially depends on two elements, known as "slippery" sequence and a complex pseudoknot structure [40]. Two functional proteinase domains, papain-like cysteine proteinase (PL2PRO) and 3C-like cysteine proteinase (3CLPRO), were identified and responsible for the proteolytic processing of

the polyprotein into 16 subunits[40-42]. PL2PRO is responsible for cleavage of ORF1a and 1b in N-terminal region and 3CLPRO is responsible for cleavage in C-terminal region. RNA-dependent RNA polymerase (RdRp) and helicase are products of proteolytic cleavage and essential components of the replicase complex which may contain other viral and cellular proteins.

5' and 3' UTR

In addition to the genomic RNA, subgenomic mRNAs are transcribed by the replicase complex. The genomic and subgenomic RNAs of coronaviruses include an identical 5' leader sequence and common 3'-ends. Recent evidence indicates that transcription-regulating sequences (TRSs) at the 5'-end of each gene represent signals which regulate the discontinuous transcription of subgenomic mRNAs [43, 44]. The leader sequence and the TRSs play important roles in the gene expression of coronavirus.

Applications of RNAi for SARS-CoV

Target sequence

RNAi has been widely used in studies of human diseases including cancers and infectious diseases. RNAi is an ideal tool to inhibit replication of viruses because siRNA can interact with specific viral RNA and silence its expression. To date, many researchers have shown that siRNAs could inhibit replication of SARS-CoV in cultured cells [45-58] (Table 2). The target sequences for silencing SARS-CoV were selected from the regions of 5'UTR, leader sequence, replicase(nsp1, nsp2, nsp3, nsp4, nsp6, RNA-dependent RNA polymerase, helicase, nsp14, and nsp16), spike, ORF3, envelope, matrix, ORF7, nucleocapsid, and 3'UTR as shown in table 2 and 3. A total of 116 siRNAs were tested and 23 siRNAs (19.8%) exhibited more than 80% reduction(Table 3). The target sites of these highly potent siRNAs were limited to following regions: leader sequence, nsp1, nsp2, nsp3, RNA-dependent RNA polymerase, helicase, nsp16 and spike.

We designed anti-SARS-CoV replicase siRNA with chemical modification(stealth siRNA, Invitrogen) and investigated the effect of siRNA on SARS-CoV replication. The targets of siSARS-1, -2, -3, -4, -5, and -6 were nsp 1, nsp 3, nsp3, 3CL proteinase, nsp 8, and nsp 14 respectively(Table 4). Vero E6 cells were transfected with siRNA duplexes 6 h before infection and infected with SARS-CoV (FFM-1 strain) at the M.O.I. of 0.01. Culture supernatants were collected 24 h after infection to purify SARS-CoV RNA. Collected RNA samples were subjected to real time RT-PCR for quantification of SARS-CoV RNA.

The results of quantitative RT-PCR showed that all of these siRNA duplexes could inhibit SARS-CoV replication in Vero E6 cells. The siSARS-3, -5, and -6 showed higher reduction activity (63%, 77%, and 69 % reduction

respectively), while the others (siSARS-1, -2, and -4) exhibited lower activity (50%, 48%, and 42% reduction respectively)(Fig. 3). Target sites of siRNAs with higher activity were nsp3, nsp8, and nsp14. Not all of siRNAs targeting the replicase gene exerted strong inhibition on viral replication, but we could identify some effective siRNAs for inhibition of SARS-CoV.

Table 2. Description of siRNA duplexes tested in the published reports.

siRNA sequence	Number	Start	End	Reference	Effectiveness(1-5)*	Target	Material	Validation
agcccaaccaactcgalct	19	28	46	55	1	S'UTR	RNA	SARS-CoV infection
gcccaaccaactcgalctc	19	29	47	45	5	Leader sequence	Plasmid vector	SARS-CoV infection
atctgtctctaaacgaac	19	54	72	55	1	S'UTR	RNA	SARS-CoV infection
gttctcctaaacgaactta	19	58	76	51	1	S'UTR	Plasmid vector	SARS-CoV infection
aacgagtaactcgcctcct	21	161	181	46	<4	S'UTR	RNA	SARS-CoV infection
gatggagagcctgttct	19	264	282	56	5	Nsp1	Plasmid vector	SARS-CoV infection
cagccctatgttcaata	19	460	478	56	5	Nsp1	Plasmid vector	SARS-CoV infection
aatfgcataccgcaatgttct	21	609	629	46	<4	Nsp1	RNA	SARS-CoV infection
gtgaactcaactcgtgagct	19	776	794	57	3	Nsp1	RNA	SARS-CoV infection
ctcactcgtgagctcaatg	19	781	799	56	5	Nsp1	Plasmid vector	SARS-CoV infection
glaccctctgattgcatc	19	849	867	57	4	Nsp2	RNA	SARS-CoV infection
gagtcgaagagaggtgtct	19	931	949	57	5	Nsp2	RNA	SARS-CoV infection
gcaactgtctacctgag	19	1209	1227	57	5	Nsp2	RNA	SARS-CoV infection
aaccttggagaagatcagct	21	2736	2756	46	<4	Nsp3	RNA	SARS-CoV infection
aatcacatllgagcttgatga	21	2787	2807	46	<4	Nsp3	RNA	SARS-CoV infection
cctccagatgaggaagaag	19	3043	3061	57	3	Nsp3	RNA	SARS-CoV infection
aacctacacclgagaagaccg	21	3251	3271	46	<4	Nsp3	RNA	SARS-CoV infection
aaggatgtcgtgtatatacac	21	4187	4207	46	<4	Nsp3	RNA	SARS-CoV infection
gggtllccttccatgtg	19	5539	5557	57	5	Nsp3	RNA	SARS-CoV infection
aaaggaccagtgactgagtt	21	5773	5793	46	<4	Nsp3	RNA	SARS-CoV infection
aagggtgtgtgataccgagtg	21	8111	8131	46	<4	Nsp3	RNA	SARS-CoV infection
atctgtagcagagacactg	19	9771	9789	51	1	Nsp4	Plasmid vector	SARS-CoV infection
aagcacgcatcttctgtctg	21	11089	11109	46	<4	Nsp6	RNA	SARS-CoV infection
caatfcaaggaggtagg	19	12714	12732	51	1	Nsp9	Plasmid vector	SARS-CoV infection
aaggatgaggaaggcaatla	21	13545	13565	46	5	RdRp	RNA	SARS-CoV infection
ggatgaggaaggcaatla	19	13547	13565	50	5	RdRp	RNA	SARS-CoV infection
ggatgaggaaggcaatla	19	13547	13565	54	3	RdRp	RNA	Transfection of target-encoding plasmids
aagagacatllataacttgg	21	13618	13638	46	<4	RdRp	RNA	SARS-CoV infection
ctggagcatttcggatg	19	14015	14033	54	1	RdRp	RNA	Transfection of target-encoding plasmids
cttactagctcgcgtctc	19	14450	14468	51	5	RdRp	Plasmid vector	SARS-CoV infection
actgtcaaacccggtaatt	19	14595	14613	54	1	RdRp	RNA	Transfection of target-encoding plasmids
aactcclatctgtagtgaag	21	14773	14793	46	<4	RdRp	RNA	SARS-CoV infection
gaatlatagcgcaggctg	19	15877	15895	51	5	RdRp	Plasmid vector	SARS-CoV infection
aaggtagctatgtgtagctg	21	16771	16791	46	<4	NTPase/HEL	RNA	SARS-CoV infection
aaggalaagtcagctcaatgc	21	17559	17579	46	5	NTPase/HEL	RNA	SARS-CoV infection
atcttaggattgcctcagc	19	17739	17757	48	5	NTPase/HEL	Plasmid vector	SARS-CoV infection
latgactatgcaatla	19	17787	17805	48	<4	NTPase/HEL	Plasmid vector	SARS-CoV infection
lgtcaaccgcctcaatg	19	17837	17855	48	<4	NTPase/HEL	Plasmid vector	SARS-CoV infection
lgtctgataagagacttla	19	17893	17911	48	<4	NTPase/HEL	Plasmid vector	SARS-CoV infection
aggacatgacctaccgtag	19	18109	18127	48	<4	Nsp14	Plasmid vector	SARS-CoV infection
aacctaccctccagctagga	21	18279	18299	46	<4	Nsp14	RNA	SARS-CoV infection
aactggcacactactgtcga	21	20858	20878	46	5	Nsp16	RNA	SARS-CoV infection
aagctcctaatatacactcaac	21	21568	21588	46	5	Spike	RNA	SARS-CoV infection
gctcctaatatacactcaac	19	21570	21588	50	5	Spike	RNA	SARS-CoV infection
aatgttacagggttcaact	21	21684	21704	46	<4	Spike	RNA	SARS-CoV infection
lgtgtatagcagcatgt	19	21857	21875	55	1	Spike	RNA	SARS-CoV infection
aaccttcttctgtcttct	21	21894	21914	53	5	Spike	RNA	Transfection of target-encoding plasmids
aagggtatcaaacctalagat	21	22083	22103	46	<4	Spike	RNA	SARS-CoV infection
gggctcaaacctalagat	19	22085	22103	55	5	Spike	RNA	SARS-CoV infection
aatcacagatgctgtgattg	21	22304	22324	46	<4	Spike	RNA	SARS-CoV infection
aagagctttagattgacaaa	21	22362	22382	53	5	Spike	RNA	Transfection of target-encoding plasmids
gagacatctcaatgtgcc	19	22849	22867	52	5	Spike	Plasmid vector	Transfection of target-encoding plasmids
aaccttaccagagttgtagtac	21	22966	22986	46	<4	Spike	RNA	SARS-CoV infection
aagatgttaactgcactgag	21	23287	23307	46	<4	Spike	RNA	SARS-CoV infection
caaggcattagctcaaatl	19	24221	24239	55	5	Spike	RNA	SARS-CoV infection
gggctaccacctatgtcc	19	24572	24590	52	5	Spike	Plasmid vector	Transfection of target-encoding plasmids
aagagctggacaagtacttca	21	24886	24906	46	<4	Spike	RNA	SARS-CoV infection
aagtaclgttcatgctacagc	21	25345	25365	46	<4	ORF3a	RNA	SARS-CoV infection
aatgcatcaacgatlagaaa	21	25614	25634	46	<4	ORF3a	RNA	SARS-CoV infection
aatlatatgagatgtggct	21	25633	25653	46	<4	ORF3a	RNA	SARS-CoV infection
aaggtagcggcattcaacac	21	25779	25799	46	<4	ORF3a	RNA	SARS-CoV infection
aaaltactacagacactggtta	21	25920	25940	46	<4	ORF3a	RNA	SARS-CoV infection
aaaatgctacatcttcaact	21	25944	25964	46	<4	ORF3a	RNA	SARS-CoV infection
aatcacacacaatcgaggctc	21	25999	26019	46	<4	ORF3a	RNA	SARS-CoV infection
argaaacaggtacgtlaalatg	21	26136	26156	46	<4	ORF3b/E	RNA	SARS-CoV infection
taalatgtaatagcgtact	21	26150	26170	47	2	ORF3b/E	RNA	Transfection of target-encoding plasmids
aatagtaatagcgtactct	21	26152	26172	46	<4	ORF3b/E	RNA	SARS-CoV infection
lctggatctctgtcagtc	21	26183	26203	47	2	E	RNA	Transfection of target-encoding plasmids
lctgattgtgctgactgct	21	26226	26246	47	2	E	RNA	Transfection of target-encoding plasmids
lgtgctactcgtcgaatatt	21	26234	26254	46	<4	E	RNA	SARS-CoV infection
aacggttactcgtactcgcg	21	26278	26298	47	3	E	RNA	Transfection of target-encoding plasmids
tgaaggattcctgactctct	21	26320	26340	47	4	E	RNA	Transfection of target-encoding plasmids
aaggagttcctgactctctgg	21	26322	26342	46	<4	E	RNA	SARS-CoV infection
agctlaaacactcctggaac	21	26429	26449	47	4	M	RNA	Transfection of target-encoding plasmids
aacctagtaataggtttccta	21	26455	26475	46	<4	M	RNA	SARS-CoV infection
lgtctgctctacagaattaa	21	26595	26615	47	2	M	RNA	Transfection of target-encoding plasmids
aatggctgtatgtaggct	21	26643	26663	46	<4	M	RNA	SARS-CoV infection
tatgtaggctgattgtggct	21	26652	26672	47	3	M	RNA	Transfection of target-encoding plasmids

Table 2. Continued

tagctactctgttctcct	21	26673	26693	47	3	M	RNA	Transfection of target-encoding plasmids
aattgtgaccagaccgctcat	21	26775	26795	46	<4	M	RNA	SARS-CoV infection
ccagaccgctcatggaagtg	21	26783	26803	47	2	M	RNA	Transfection of target-encoding plasmids
ttgcaatggccggacaccc	21	26839	26859	47	2	M	RNA	Transfection of target-encoding plasmids
aagagatcaactgtgctacat	21	26891	26911	46	<4	M	RNA	SARS-CoV infection
agagatcaactgtgctacat	21	26892	26912	47	3	M	RNA	Transfection of target-encoding plasmids
cgctgcagcgtgtaggcactg	21	26942	26962	47	3	M	RNA	Transfection of target-encoding plasmids
ttgctgcalacaaccgclacc	21	26972	26992	47	4	M	RNA	Transfection of target-encoding plasmids
aaccgctaccglatggaaac	21	26983	27003	46	<4	M	RNA	SARS-CoV infection
aaactataaataaacacaga	21	27000	27020	47	2	M	RNA	Transfection of target-encoding plasmids
aaactgtccatcaggaacat	21	27370	27390	46	<4	ORF7a	RNA	SARS-CoV infection
aaactgtccatcagcacactt	21	27440	27460	46	<4	ORF7a	RNA	SARS-CoV infection
aagagctctactcgcacttt	21	27556	27576	46	<4	ORF7a	RNA	SARS-CoV infection
glctaaaagcaatgaaac	19	27767	27785	51	1	ORF7b	Plasmid vector	SARS-CoV infection
galaatggaccccaatcaaac	21	28126	28146	47	4	N	RNA	Transfection of target-encoding plasmids
gllgaccacagatcaac	19	28175	28193	49	3	N	Plasmid vector	Transfection of target-encoding plasmids
aaclgacaataaccagaatgg	21	28191	28211	46	<4	N	RNA	SARS-CoV infection
aaggccaaaacagccgacc	21	28227	28247	47	2	N	RNA	Transfection of target-encoding plasmids
aataatactgctctgggttc	21	28261	28281	47	3	N	RNA	Transfection of target-encoding plasmids
aaggaggaaactagattccct	21	28303	28323	47	3	N	RNA	Transfection of target-encoding plasmids
ggaggaacttagattccct	19	28305	28323	58	4	N	Plasmid vector	Transfection of target-encoding plasmids
aaattgctctaccgaagag	21	28370	28390	46	<4	N	RNA	SARS-CoV infection
aagagctaccgacaggtctg	21	28386	28406	47	1	N	RNA	Transfection of target-encoding plasmids
aacaaagaggcatcgtatgg	21	28498	28518	47	2	N	RNA	Transfection of target-encoding plasmids
ggcatcgtatgggttgaact	21	28507	28527	58	5	N	Plasmid vector	Transfection of target-encoding plasmids
aalacaccaagaccacatt	21	28540	28560	47	2	N	RNA	Transfection of target-encoding plasmids
aatcctaaalaacaaigtctgc	21	28570	28590	47	3	N	RNA	Transfection of target-encoding plasmids
lcaaggaacaacttgccaaa	21	28608	28628	47	3	N	RNA	Transfection of target-encoding plasmids
gaaatcaactcctggcag	19	28706	28724	58	3	N	Plasmid vector	Transfection of target-encoding plasmids
aaccagcttgagagcaaatgt	21	28804	28824	46	<4	N	RNA	SARS-CoV infection
tgaggaacttaaaagcctcg	21	28878	28898	47	2	N	RNA	Transfection of target-encoding plasmids
aacgtaactgccaacaaacagt	21	28904	28924	47	2	N	RNA	Transfection of target-encoding plasmids
aacagtaacactcactcaag	21	28919	28939	46	<4	N	RNA	SARS-CoV infection
gtcactcaagcattgggaga	21	28930	28950	58	3	N	Plasmid vector	Transfection of target-encoding plasmids
aagcacatlgacgcatacaaa	21	29185	29205	46	<4	N	RNA	SARS-CoV infection
aagactgatgaagctcagcct	21	29245	29265	46	<4	N	RNA	SARS-CoV infection
ttcgcaattcgtttacg	19	29432	29450	55	1	3'UTR	RNA	SARS-CoV infection
cgtlaactaacagcacaag	19	29484	29502	55	3	3'UTR	RNA	SARS-CoV infection

* Effectiveness 1 : 0-20% reduction, 2 : 20-40% reduction, 3 : 40-60% reduction, 4 : 60-80% reduction, 5 : 80-100% reduction

Table 3. Effectiveness of tested siRNAs.

Target of siRNA	Synthetic RNA			Plasmid vector			Total		
	The number of tested siRNAs	The number of effective siRNAs (>80% reduction)	Ratio of effective siRNAs (%)	The number of tested siRNAs	The number of effective siRNAs (>80% reduction)	Ratio of effective siRNAs (%)	The number of tested siRNAs	The number of effective siRNAs (>80% reduction)	Ratio of effective siRNAs (%)
5'UTR/Leader	3	0	0.0	2	1	50.0	5	1	20.0
Replicase	25	7	28.0	12	6	50.0	37	13	35.1
Spike	13	6	46.2	2	2	100.0	15	8	53.3
ORF3	10	0	0.0	0	0	NC	10	0	0.0
E	6	0	0.0	0	0	NC	6	0	0.0
M	15	0	0.0	0	0	NC	15	0	0.0
ORF7	3	0	0.0	1	0	0.0	4	0	0.0
N	17	0	0.0	5	1	20.0	22	1	4.5
3'UTR	2	0	0.0	0	0	NC	2	0	0.0
Total	94	13	13.8	22	10	45.5	116	23	19.8

Table 4. Description of siRNAs tested in our study.

siRNA	sequence	Number	Start	End	Effectiveness(1-5)	Target	Material	Validation
siSARS-1	gctatggcatcgatctaaagtctta	25	668	692	3	Nsp1	RNA	SARS-CoV infection
siSARS-2	gccattaaatgtgtgacatcgta	25	3313	3337	3	Nsp3	RNA	SARS-CoV infection
siSARS-3	gccacacgctgtgagtgtaacata	25	7447	7471	4	Nsp3	RNA	SARS-CoV infection
siSARS-4	gctggcaatgttcaactcgtgta	25	10192	10216	3	3CL pro	RNA	SARS-CoV infection
siSARS-5	gctctaagagccaactcagctgta	25	12583	12607	4	Nsp8	RNA	SARS-CoV infection
siSARS-6	gcacatgtgctagtgtgatgta	25	18768	18792	4	Nsp14	RNA	SARS-CoV infection

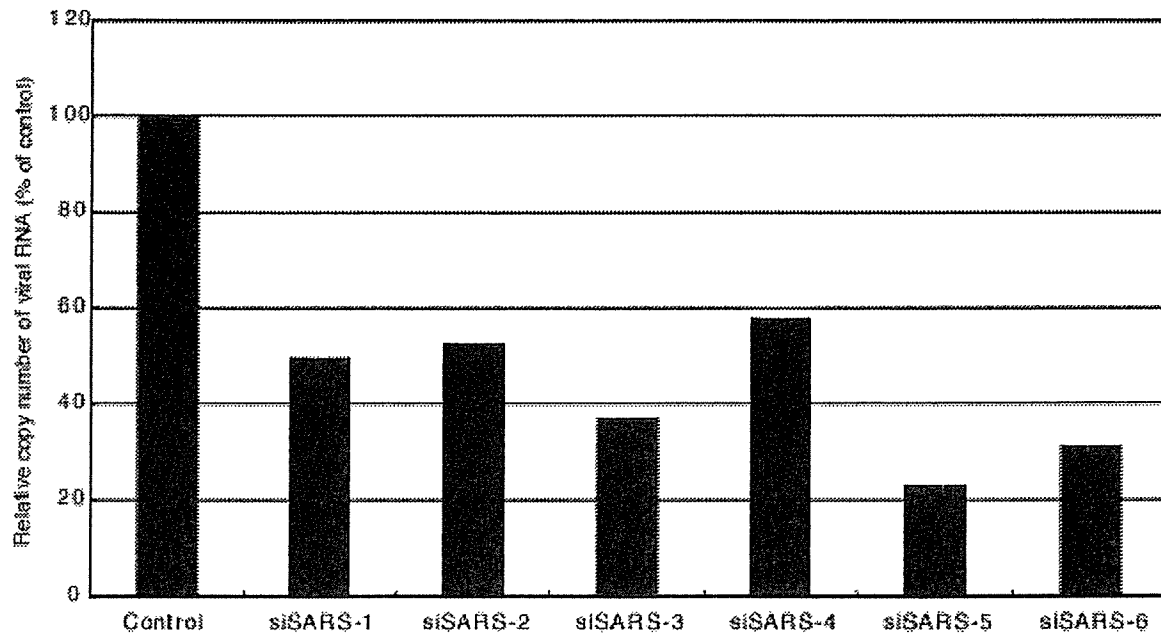


Figure 3. Inhibition of SARS-CoV replication by siRNAs targeting ORF1. We designed anti-SARS-CoV replicase siRNA with chemical modification and investigated the effect of siRNA on SARS-CoV replication by real-time RT-PCR. The targets of siSARS-1, -2, -3, -4, -5, and -6 were nsp 1, nsp 3, nsp3, 3CL proteinase, nsp 8, and nsp 14 respectively. The siSARS-3, -5, and -6 showed higher reduction activity (63%, 77%, and 69 % reduction respectively), while the others (siSARS-1, -2, and -4) exhibited lower activity (50%, 48%, and 42% reduction respectively).

Targeting leader sequence

Li et al. showed that siRNA targeting the leader sequence of SARS-CoV could strongly inhibit virus replication[45]. The common feature of the coronaviruses is the similar discontinuous transcription of subgenomic mRNA regulated by the pretranscribed leader sequence and the specific TRSs in the genome[43, 44]. It was shown that targeting the leader sequence in the mRNA of SARS-CoV structural proteins was a powerful way to control virus replication because the leader sequence contributes to the regulation of virus gene expression and because the leader sequence remained almost identical in all the strains of SARS-CoV identified.

Targeting nsp1,2 and 3

Ni et al. reported that shRNA-expression plasmids targeting nsp1 gene in replicase complex could effectively block SARS-CoV replication[56]. They demonstrated that the siRNA against nsp1 resulted in the global reduction of viral gene expression including spike and nucleocapsid. Since the sequence of nsp1 is highly conserved, it is reasonable to select this sequence as a target to control SARS-CoV infection. He and Zheng et al. tested six siRNAs targeting

different sites in the nsp1, nsp2 and nsp3, and showed that three siRNAs targeting nsp2 had stronger inhibitory effect on SARS-CoV replication than those against nsp1 and nsp3[57]. These siRNAs could protect cells from SARS-CoV-induced cytopathic effect and inhibit production of SARS-CoV antigens. The reduction of the viral genomic RNA copies was 92.5%, 89.6%, and 85.8% in the cells transfected with SARSi-4, SARSi-2 and SARSi-2 all of which targeted nsp2 region of SARS-CoV. They transfected FRhk-4 cells with a combination of 2 or 3 effective siRNAs to check whether siRNAs could show synergistic inhibition of viral replication. However, no obvious synergistic effects were observed from any of these combinations.

Targeting RNA-dependent RNA polymerase

RNA-dependent RNA polymerase plays a critical role in synthesis of viral RNAs and it is logically acceptable to use siRNAs targeting RNA-dependent RNA polymerase for blocking SARS-CoV replication. Two groups designed various kinds of siRNAs including that specific to RNA-dependent RNA polymerase and both groups proved that siRNAs targeting RNA polymerase could remarkably inhibit SARS-CoV replication[46, 51]. Zheng et al. transfected the cells with seven combinations of the active siRNAs including polymerase-targeting siRNA duplexes to test the hypothesis that multiple active siRNAs could improve the prophylactic and therapeutic effects. Interestingly, all combinations presented much improved potency of the prophylactic and therapeutic effects. [46]

Targeting spike

Several groups tested the effectiveness of siRNAs which have a sequence corresponding to spike gene of SARS-CoV[46, 50, 52, 53, 55]. Spike protein can be a good target for inhibition of SARS-CoV replication because SARS-CoV spike protein plays a critical role during infection. Qin et al. showed that the siRNAs transcribed in vitro were effective in silencing S glycoprotein expression;[Qin, 2004 #1901], and Zhang et al. showed that DNA vector-driven siRNA could selectively silence S gene expression in Vero E6 cells infected with SARS-CoV[52]. Wu et al. demonstrated that synthetic siRNA duplexes directed against spike sequence could inhibit SARS-CoV replication in Vero E6 cells more strongly than siRNAs targeting the other regions[55].

Targeting genes encoded in subgenomic RNA except spike

Many researchers tested various kinds of siRNAs and showed that siRNAs targeting leader sequence, replicase and spike could inhibit SARS-CoV

replication[45, 46, 50, 52, 53, 55, 56]. However, it was difficult to find strongly active siRNA in the genes encoded in subgenomic RNAs excepting spike. Almost all of the siRNAs directed against ORF3, envelope, matrix, ORF7a, and nucleocapsid were weak in blocking virus replication[46, 51](Table 2 and 3). These results suggest that genomic RNA disruption may be the major mechanism in inhibition of viral replication by active siRNAs, rather than viral protein knockdown. Zheng showed that the effective siRNA targeting spike region could reduce both viral titer and viral genome copy number by destruction of genomic RNA , in addition to knockdown of spike protein expression[46].

Using siRNA against SARS-CoV in vivo

Many reports indicated that selected siRNA could effectively inhibit SARS-CoV replication in vitro, but siRNA-mediated inhibition of SARS had scarcely been shown in animal models. Li et al. demonstrated for the first time that siRNAs have a significant effect on suppression of SARS-like symptoms in a macaque model[50]. Importantly, this approach was shown to be effective both prophylactically and therapeutically with no adverse effects.

The authors selected two siRNAs targeting RNA-dependent RNA polymerase and spike which were shown to be effective in cultured cells. They first tested two solutions for intranasal delivery of the siRNA duplexes and the luciferase reporter plasmid into mouse lungs, and identified D5W (a glucose solution) as the reagent with higher delivery efficiency.

They also used this D5W reagent for delivery of siRNAs to Rhesus macaque lungs. Although concern remains over whether the macaque SARS models are clinically relevant, the Rhesus macaque model used in this study mirrors the sequence of pathogenesis in humans with SARS and the cynomolgus macaque SARS model[3, 59]. Clear clinical relevance of this model could be found in most of the parameters studied, including elevated body temperature, lung pathology and SARS-CoV antigen detection in type I pneumocytes, type II pneumocytes and macrophages. Therefore, this Rhesus macaque model is one of the ideal system for evaluation of new therapeutic agents against SARS.

Using this model, Li et al. observed that all the monkeys in the siRNA-treated groups showed less severe symptoms than the control groups. For instance, during the 20-day period of observation after administration of the siRNAs, the treated monkeys kept an average body temperature lower than 38.9 °C while the control monkeys had an average of body temperature above 39.1 °C. All of the three siRNA-treated groups showed relatively mild severity of acute diffuse alveolar damage (DAD) and none showed lung damage beyond a score of ++, whereas the control animals suffered from severe DAD with a score of +++. Furthermore, it was shown that three of the four animals

in each of the siRNA-treated groups were free of SARS-CoV RNA in the oropharyngeal swab specimens. These results indicated that the siRNA treatments blocked the spread of SARS-CoV within the lungs and suppressed SARS-like symptoms. The total accumulated dosage of siRNA administered to individual macaques ranged from 10 mg/kg to 40 mg/kg and did not cause any visible differences in appearance, behavior or sign of organ damage, which indicated that siRNA was safe for prophylactic and therapeutic treatments *in vivo*. This study has important implications on strategies for combating emerging infectious diseases such as SARS.

RNAi and viral diseases other than SARS-CoV

RNAi has been developed into a widely used technique, and many researchers have reported inhibition of virus replication by means of RNAi for various viruses, including human immunodeficiency virus type 1, hepatitis B virus, hepatitis C virus, dengue virus, poliovirus and influenza A virus[26-30, 60-69].

Treatment of HIV-infected patients with combination of antiviral drugs is effective, but there are problems concerning chronic toxicity of drugs and the emergence of drug-resistant HIV-1 strains. Various research groups have investigated effectiveness of RNAi on HIV-1 in search for alternative strategies to block HIV-1 replication. Target sequences of siRNA in these reports were the structural proteins Gag, Pol and Env, and the regulatory proteins Tat and Rev, and two accessory proteins Nef and Vif [26-28, 70-73].

Although it was demonstrated that HIV-1 replication could be inhibited by RNAi, the question remains whether siRNA targets the incoming genomic RNA, the newly synthesized transcripts or both. Evidences have been obtained suggesting that the incoming genomic RNA is indeed targeted for siRNA-mediated destruction[26, 27, 71]. In these studies the amount of integrated provirus was found to be reduced when cells were pre-treated with siRNAs. However, it was reported in the other studies that the level of proviral DNA was not decreased by treatment of siRNA[70, 72, 74]. It is possible that different siRNAs induce different effects. It seems that targeting the incoming genomic RNA can explain only a small part of the inhibition and that viral transcripts are much more susceptible to RNAi-mediated degradation. This is in agreement with studies on the other retroviruses, which revealed that the incoming RNA was protected against RNAi-mediated degradation by the nucleocapsid [70].

In addition to targeting the viral RNA, an alternative way to inhibit virus replication by RNAi is to silence the expression of cellular genes that are required for viral replication. The targets of siRNA against host factors included CD4, CCR5, CXCR4 and p65 subunit of NF-kappa B[26, 74-76]. *In vivo* suppression of CD4 and NF kappa B is limited because of the important

functions of these molecules. The HIV-1 co-receptor CCR5 may be a more suitable target because an inactivating mutation in the CCR5 gene is compatible with normal life. By using a lentivirus-based vector system carrying the siRNA expression cassette against CCR5, expression of CCR5 on the surface of peripheral blood T lymphocytes was reduced by 10-fold, which resulted in a 3- to 7-fold reduction in the number of infected cells [76]. Compared with direct HIV-RNA targeting, the indirect approach silencing a viral receptor by siRNA appears less effective in blocking HIV-1 replication.

Several groups also investigated the effects of siRNA targeting host factors to control replication of viruses other than HIV-1. Gaggar et al. [77] used CD46-specific siRNAs to show the role of CD46 as a cellular attachment receptor for most group B adenoviruses. Similarly, the role of the ubiquitous glucose transporter GLUT-1 as a component of the receptor for the human T cell leukemia virus (HTLV) was demonstrated by silencing GLUT-1 expression with siRNAs specific for the 3'NC region of GLUT-1 mRNA [78]. Adenovirus vectors encoding siRNAs have been used to confirm the role of putative cellular cofactors for HCV. HCV replication in Huh-7 cells was substantially inhibited by silencing either La (a protein potentially involved in HCV internal initiation of translation), polypyrimidine tract binding protein (a protein interacting with both 5'- and 3'-NC regions), or gamma subunit of eukaryotic initiation factors 2B (a cofactor of HCV cap-independent translation) [79]. Similarly, targeting the human RNA helicase p68 by specific siRNAs reduced the transcription of negative strand of HCV RNA [80]. Moreover, caveolin-1 knockdown by RNAi reduced human coronavirus 229E infection, because this virus binds to CD13 in rafts and enters the cell through caveolae [81].

On the study of SARS-CoV, there has been no report using siRNA which targets cellular factors required for multiplication such as ACE2. Combining siRNAs targeting SARS-CoV RNA with those targeting host factors may result in stronger inhibition of virus replication.

Improvement of RNAi technology for application

Chemical modification

Although duplex RNA is more resistant to nuclease than single-stranded RNA, unmodified siRNAs are rapidly degraded when added into culture medium containing serum or when administered intravenously in mammals. Degradation of siRNA duplex can be delayed or avoided by chemical modification of the oligonucleotide. Various kinds of chemical modifications that confer nuclease resistance have been developed in single-stranded antisense oligonucleotides and many of these modifications can be used in siRNA. The chemical modification in siRNA duplexes include boranophosphate modification,

phosphorothioate modification, 2'-O-methyl modification, 2'-fluoro modification, locked nucleic acids (LNAs) and cholesterol conjugation.

Phosphorothioate or boranophosphate modification of the inter-nucleoside linkage improves nuclease stability. Unfortunately, it is difficult to synthesize boranophosphate-modified RNA by standard chemical synthesis methods. Instead, borano-modified bases were incorporated in RNA using an *in vitro* transcription method[82], which made site-selective placement of this modification difficult. In contrast to boranophosphate modification, phosphorothioate modification can be easily placed in siRNA duplexes at any position and will increase stability of siRNA duplexes in serum or other nuclease sources[83-86].

Modification of the 2' position of the ribose can increase binding activity of both strands in siRNA duplexes (can increase T_m value) and confers nuclease resistance. The 2'-O-methyl RNA is a naturally occurring base that exists in mammalian ribosomal RNA. Alternating modification by 2'-O-methyl substitution retains potency and confers substantial nuclease resistance[87].

The 2'-fluoro modification has also been used with good results and this modification at pyrimidine bases can be combined with 2'-O-methyl purines[83-85, 88].

LNA are a different class of 2' modification in which a methylene bridge connects the 2'-O with the 4'-C of the ribose. LNA can improve binding activity of both strands and increase resistance against nuclease. LNAs can be used in siRNA duplexes but placement of modified nucleic acids to achieve best activity is more restricted than nucleic acids containing 2'-O-methyl or 2'-fluoro modification [54, 85, 89].

Cholesterol-conjugated siRNA (chol-siRNA) was shown to be effective in silencing an endogenous gene by systemic administration [90]. The conjugation of cholesterol to the 3' end of the sense strand of a siRNA molecule did not result in a significant loss of gene-silencing activity in cell culture. Moreover, unlike unconjugated siRNAs, chol-siRNA targeting luciferase showed reduction of luciferase activity in HeLa cells with a half-maximal inhibitory concentration (IC_{50}) of about 200 nM in the absence of transfection reagents. Although no detectable amounts of unconjugated siRNAs were observed in tissue samples, significant levels of chol-siRNAs were detected in liver, heart, kidney, adipose, and lung tissue samples.

In the study of siRNA-mediated inhibition of SARS-CoV replication, siRNAs with chemical modification have not been tested yet. We investigated the effect of chemically modified siRNAs targeting replicase gene and found that some of these siRNAs could block replication of SARS-CoV.

It may be possible to develop siRNAs with higher anti-SARS-CoV activity by optimization of target sequence and chemical modification especially for *in vivo* use.

Delivery of siRNA

Cationic liposomes and polymers are two major classes of nonviral carriers for siRNA delivery and they were used in the majority of studies on inhibition of SARS-CoV replication by siRNA. Both of them are positively charged and can form complexes with negatively charged siRNA. The complexes of siRNA and carriers can be condensed into nanoparticles, which allows efficient uptake of siRNA by endocytosis.

A different type of lipid particle was employed by Morrissey and colleagues to deliver anti-HBV siRNAs in a mouse hepatitis B model system[91]. The stable nucleic acid lipid particles (SNALP) consists of lipid bilayer containing cationic and fusogenic lipids that enables the cellular uptake and endosomal release of nucleic acids. SNALPs are also coated with a diffusible polyethylene glycol-lipid(PEG-lipid) conjugate that provides a neutral, hydrophilic exterior. Surface coating also shields the cationic bilayer *in vivo*, preventing rapid systemic clearance. Upon administration, PEG-lipid conjugate dissociates from the SNALP and the particle was transformed into a transfection-competent entity.

For *in vivo* study of anti-SARS-CoV siRNA, D5W solution (5% D-glucose in water) was used as a carrier of siRNA duplex instead of cationic liposomes[50]. It may be necessary to select the best carrier according to the target organ.

Vectors for RNAi

At the beginning of RNAi research, only the synthesized short RNA duplexes could be used to knock down the expression of target genes. Several groups developed plasmid-based systems for RNAi and these expression systems have been widely used for gene silencing. It is shown in table 2 and 3 that plasmid-based systems could knock down more efficiently than the duplexes of synthesized short RNA. Higher ratio of effective siRNA in vector-based system may be due to (1) higher stability of DNA than RNA and (2) higher copy number of siRNA in vector-based expression systems than that in synthesized RNA. The intracellular production of siRNAs can provide long-term resistance to viral infection. However, the limitation of plasmid-based system is the dependence on transfection. Only certain cell lines can be transfected and efficient transfection of primary cells is virtually impossible. To overcome the problems in plasmid-based system, various kinds of virus-based vectors have been developed which include adenovirus vector, adeno-associated virus vector, retrovirus vector and lentivirus vector. The best vector for delivering an siRNA template would be a lentivirus vector derived from HIV-1, because lentivirus vectors can stably infect nondividing cells. Lentiviral vectors have proven to be effective in expressing genes within

multiple lineages over prolonged periods. To date, there has been no report on inhibition of SARS-CoV replication with lentivirus-based vectors, but it is likely that siRNAs mediated by lentivirus vectors can inhibit replication of SARS-CoV more efficiently than those mediated by plasmid vectors.

Concluding remarks

SARS-CoV was identified as the etiological agent of SARS and was shown to be a novel member in the coronavirus family by phylogenetic analysis. A number of prophylactic and therapeutic strategies for SARS were reported which included glycyrrhizin, HIV-1 protease inhibitors, and interferons, but specific treatment for this disease has not been established yet. RNAi is a recently discovered antiviral mechanism in plant and animal cells that induces specific degradation of target RNA. RNAi is considered as a good alternative approach to the infectious diseases such as SARS because of its specificity and efficiency. SARS-CoV replication could be potently inhibited by siRNA targeting leader sequence, nsp1, nsp2, nsp3, RNA-dependent RNA polymerase, helicase, nsp16 and spike. Moreover, the recent work demonstrated that selected siRNAs could suppress SARS-CoV replication and SARS-like symptoms in Rhesus macaque model. RNAi technology will be improved by development of new siRNAs with chemical modification, new reagents for efficient delivery of siRNAs, and virus-based vector systems for stable and efficient degradation of target mRNA. Treatment of infectious diseases including SARS with RNAi is promising and in the future RNAi may be used in human for treatment of diseases after solving the present problems in utilization of RNAi in vivo.

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