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)*	T arg et	Material	Varidation
agccaaccaacctcgatct	19	28	46	55	1	5'UTR	RNA	SARS-CoV infection
gccaaccaacctcgatctc	19	29	47	45	5	Leader sequence	Plasmid vector	SARS-CoV infection
atctgttctctaaacgaac	19	54	72	55	1	5'UTR	RNA	SARS-CoV infection
gttctctaaacgaacttta	19	58	76	51	1	5'UTR	Plasmid vector	SARS-CoV infection
aacgagtaactcgtcccictt	21	161	181	46	<4	5'UTR	RNA	SARS-CoV infection
gatggagagccttgttctt	19	264	282	56	5	Nsp1	Plasmid vector	SARS-CoV infection
cagccctatgtgttcatta	19	460	478	56	5	Nsp1	Plasmid vector	SARS-CoV infection
aattgcataccgcaatgttct	21	609	629	46	<4	Nsp1	RNA	SARS-CoV infection
gtgaactcactcgtgagct	19	776	794	57	3	Nsp1	RNA	SARS-CoV infection
ctcactcgtgagctcaatg	19	781	799	56	5	Nsp1	Plasmid vector	SARS-CoV infection
gtaccctcttgattgcatc	19	849	867	57	4	Nsp2	RNA	SARS-CoV infection
gagtcgaagagaggtgtct	19	931	949	57	5	Nsp2	RNA	SARS-CoV infection
gcacttgtctaccttgatg	19	1209	1227	57	5	Nsp2	RNA	SARS-CoV infection
aaccttggagaagatactgt	21	2736	2756	46	<4	Nsp3	RNA	SARS-CoV infection
aatcacatttgagcttgatga	21	2787	2807	46	<4	Nsp3	RNA	SARS-CoV infection
cctccagatgaggaagaag	19	3043	3061	57	3	Nsp3	RNA	SARS-CoV infection
aacctacacctgaagaaccag	21	3251	3271	46	<4	Nsp3	RNA	SARS-CoV infection
aaggatgtgctggttatacac	21	4187	4207	46	<4	Nsp3	RNA	SARS-CoV infection
ggtgtttccattccatgtg	19	5539	5557	57	5	Nsp3	RNA	SARS-CoV infection
aaaggaccagtgactgatgtt	21	5773	5793	46	<4	Nsp3	RNA	SARS-CoV infection
aagggtgttgataccgatg	21	8111	8131	46	<4	Nsp3	RNA	SARS-CoV infection
attgcgtagcgagacactg	19	9771	9789	51	1	Nsp4	Plasmid vector	SARS-CoV infection
aagcacgcattcttgtgcttg	21	11089	11109	46	<4	Nsp6	RNA	SARS-CoV infection
caattcgaagggaggtagg	19	12714	12732	51	1	Nsp9	Plasmid vector	SARS-CoV infection
aaggatgaggaaggcaattta	21	13545	13565	46	5	RdRp	RNA	SARS-CoV infection
ggatgaggaaggcaattta	19	13547	13565	50	5	RdRp	RNA	SARS-CoV infection
ggatgaggaaggcaattta	19	13547	13565	54	3	RdRp	RNA	Transfection of target-encoding plasmids
aagagactatttataacttgg	21	13618	13638	46	<4	RdRp	RNA	SARS-CoV infection
ctggtacgatttcggtgat	19	14015	14033	54	1	RdRp	RNA	Transfection of target-encoding plasmids
cttacatagctcgcgtctc	19	14450	14468	51	5	RdRp	Plasmid vector	SARS-CoV infection
actgtcaaacccggtaatt	19	14595	14613	54	1	RdRp	RNA	Transfection of target-encoding plasmids
aactcctattcgtagttgaag	21	14773	14793	46	<4	RdRp	RNA	SARS-CoV infection
gaatattaggcgcaggctg	19	15877	15895	51	5	RdRp	Plasmid vector	SARS-CoV infection
aaggtgactatggtgatgctg	21	16771	16791	46	<4	NTPase/HEL	RNA	SARS-CoV infection
aaggataagtcagctcaatgc	21	17559	17579	46	5	NTPase/HEL	RNA	SARS-CoV infection
atcttaggattgcctacgc	19	17739	17757	48	5	NTPase/HEL	Plasmid vector	SARS-CoV infection
tatgactatgtcatattca	19	17787	17805	48	<4	NTPase/HEL	Plasmid vector	SARS-CoV infection
tgtcaaccgcttcaatgtg	19	17837	17855	48	<4	NTPase/HEL	Plasmid vector	SARS-CoV infection
tgtctgatagagatcttta	19	17893	17911	48	<4	NTPase/HEL	Plasmid vector	SARS-CoV infection
aggacatgacctaccgtag	19	18109	18127	48	<4	Nsp14	Plasmid vector	SARS-CoV infection
aacctacctctccagctagga	21	18279	18299	46	<4	Nsp14	RNA	SARS-CoV infection
aactggcacactacttgtcga	21	20858	20878	46	5	Nsp16	RNA	SARS-CoV infection
aagctcctaattacactcaac	21	21568	21588	46	5	Spike	RNA	SARS-CoV infection
gctcctaattacactcaac	19	21570	21588	50	5	Spike	RNA	SARS-CoV infection
aatgttacagggtttcatact	21	21684	21704	46	<4	Spike	RNA	SARS-CoV infection
tgttgttatacgagcatgt	19	21857	21875	55	1	Spike	RNA	SARS-CoV infection
aaccctttctttgctgtttct	21	21894	21914	53	5	Spike	RNA	Transfection of target-encoding plasmids
aagggctatcaacctatagat	21	22083	22103	46	<4	Spike	RNA	SARS-CoV infection
gggctatcaacctatagat	19	22085	22103	55	5	Spike	RNA	SARS-CoV infection
aatcacagatgctgttgattg	21	22304	22324	46	<4	Spike	RNA	SARS-CoV infection
aagagcttgagattgacaaa	21	22362	22382	53	5	Spike	RNA	Transfection of target-encoding plasmids
gagacatatctaatgtgcc	19	22849	22867	52	5	Spike	Plasmid vector	Transfection of target-encoding plasmids
aaccttacagagttgtagtac	21	22966	22986	46	<4	Spike	RNA	SARS-CoV infection
aagatgttaactgcactgatg	21	23287	23307	46	<4	Spike	RNA	SARS-CoV infection
caaggcgattagtcaaatt	19	24221	24239	55	5	Spike	RNA	SARS-CoV infection
gggctaccaccttatgtcc	19	24572	24590	52	5	Spike	Plasmid vector	Transfection of target-encoding plasmids
aagagctggacaagtacttca	21	24886	24906	46	<4	Spike	RNA	SARS-CoV infection
aagtactgttcatgctacagc	21	25345	25365	46	<4	ORF3a	RNA	SARS-CoV infection
aatgcatcaacgcatgtagaa	21	25614	25634	46	<4	ORF3a	RNA	SARS-CoV infection
aattattatgagatgttggct	21	25633	25653	46	<4	ORF3a	RNA	SARS-CoV infection
aaggtgacggcatttcaacac	21	25779	25799	46	<4	ORF3a	RNA	SARS-CoV infection
aaattactacagacactggta	21	25920	25940	46	<4	ORF3a	RNA	SARS-CoV infection
aaaatgctacattcttcatct	21	25944	25964	46	<4	ORF3a	RNA	SARS-CoV infection
aatacacacaatcgacggctc	21	25999	26019	46	<4	ORF3a	RNA	SARS-CoV infection
aagaaacaggtacgttaatag	21	26136	26156	46	<4	ORF3b/E	RNA	SARS-CoV infection
ttaatagttaatagcgtactt	21	26150	26170	47	2	ORF3b/E	RNA	Transfection of target-encoding plasmids
aatagttaatagcgtacttct	21	26152	26172	46	<4	ORF3b/E	RNA	SARS-CoV infection
ttcgtggtattcttgctagtc	21	26183	26203	47	2	E	RNA	Transfection of target-encoding plasmids
ttcgattgtgtgcgtactgct	21	26226	26246	47	2	E	RNA	Transfection of target-encoding plasmids
tgtgcgtactgctgcaatatt	21	26234	26254	46	<4	E	RNA	SARS-CoV infection
aacggtttacgtctactcgcg	21	26278	26298	47	3	E	RNA	Transfection of target-encoding plasmids
tgaaggagttcctgatcttct	21	26320	26340	47	4	E	RNA	Transfection of target-encoding plasmids
aaggagttcctgatcttctgg	21	26322	26342	46	<4	E	RNA	SARS-CoV infection
agcttaaacaactcctggaac	21	26429	26449	47	4	M	RNA	Transfection of target-encoding plasmids
aacctagtaataggtttccta	21	26455	26475	46	<4	M	RNA	SARS-CoV infection
tgctgctgtctacagaattaa	21	26595	26615	47	2	M	RNA	Transfection of target-encoding plasmids
aatggcttgtattgtaggctt	21	26643	26663	46	<4	M	RNA	SARS-CoV infection
tattgtaggcttgatgtggct	21	26652	26672	47	3	M	RNA	Transfection of target-encoding plasmids

Table 2. Continued

tagctacttcgttgcttcctt	21	26673	26693	47	3	M	RNA	Transfection of target-encoding plasmids
aattgtgaccagaccgctcat	21	26775	26795	46	<4	M	RNA	SARS-CoV infection
ccagaccgctcatggaaagtg	21	26783	26803	47	2	M	RNA	Transfection of target-encoding plasmids
ttgcgaatggccggacactcc	21	26839	26859	47	2	M	RNA	Transfection of target-encoding plasmids
aagagatcactgtggctacat	21	26891	26911	46	<4	M	RNA	SARS-CoV infection
agagatcactgtggctacatc	21	26892	26912	47	3	M	RNA	Transfection of target-encoding plasmids
cgtcgcagcgtgtaggcactg	21	26942	26962	47	3	M	RNA	Transfection of target-encoding plasmids
ttgctgcatacaaccgctacc	21	26972	26992	47	4	M	RNA	Transfection of target-encoding plasmids
aaccgctaccgtattggaaac	21	26983	27003	46	<4	M	RNA	SARS-CoV infection
aaactataaattaaatacaga	21	27000	27020	47	2	M	RNA	Transfection of target-encoding plasmids
aaccttgcccatcaggaacat	21	27370	27390	46	<4	ORF7a	RNA	SARS-CoV infection
aacttgcactagcacacactt	21	27440	27460	46	<4	ORF7a	RNA	SARS-CoV infection
aagagctctactcgccacttt	21	27556	27576	46	<4	ORF7a	RNA	SARS-CoV infection
gtctaaacgaacatgaaac	19	27767	27785	51	1	ORF7b	Plasmid vector	SARS-CoV infection
gataatggaccccaatcaaac	21	28126	28146	47	4	N	RNA	Transfection of target-encoding plasmids
gtggacccacagattcaac	19	28175	28193	49	3	N	Plasmid vector	Transfection of target-encoding plasmids
aactgacaataaccagaatgg	21	28191	28211	46	<4	N	RNA	SARS-CoV infection
aaggccaaaacagcgccgacc	21	28227	28247	47	2	N	RNA	Transfection of target-encoding plasmids
aataatactgcgtcttggttc	21	28261	28281	47	3	N	RNA	Transfection of target-encoding plasmids
aaggaggaacttagattccct	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
aaattggctactaccgaagag	21	28370	28390	46	<4	N	RNA	SARS-CoV infection
aagagctacccgacgagttcg	21	28386	28406	47	1	N	RNA	Transfection of target-encoding plasmids
aacaaagaaggcatcgtatgg	21	28498	28518	47	2	N	RNA	Transfection of target-encoding plasmids
ggcatcgtatgggttgcaact	21	28507	28527	58	5	N	Plasmid vector	Transfection of target-encoding plasmids
aatacacccaaagaccacatt	21	28540	28560	47	2	N	RNA	Transfection of target-encoding plasmids
aatcctaataacaatgctgcc	21	28570	28590	47	3	N	RNA	Transfection of target-encoding plasmids
tcaaggaacaacattgccaaa	21	28608	28628	47	3	N	RNA	Transfection of target-encoding plasmids
gaaattcaactcctggcag	19	28706	28724	58	3	N	Plasmid vector	Transfection of target-encoding plasmids
aaccagcttgagagcaaagtt	21	28804	28824	46	<4	N	RNA	SARS-CoV infection
tgaggcatctaaaaagcctcg	21	28878	28898	47	2	N	RNA	Transfection of target-encoding plasmids
aacgtactgccacaaaacagt	21	28904	28924	47	2	N	RNA	Transfection of target-encoding plasmids
aacagtacaacgtcactcaag	21	28919	28939	46	<4	N	RNA	SARS-CoV infection
gtcactcaagcatttgggaga	21	28930	28950	58	3	N	Plasmid vector	Transfection of target-encoding plasmids
aagcacattgacgcatacaaa	21	29185	29205	46	<4	N	RNA	SARS-CoV infection
aagactgatgaagctcagcct	21	29245	29265	46	<4	N	RNA	SARS-CoV infection
tttcgcaattccgtttacg	19	29432	29450	55	1	3'UTR	RNA	SARS-CoV infection
cgtaactaaacagcacaag	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	gccattaaatgtgttgacatcgtta	25	3313	3337	3	Nsp3	RNA	SARS-CoV infection
siSARS-3	gccacacgcgttgagtgtacaacta	25	7447	7471	4	Nsp3	RNA	SARS-CoV infection
siSARS-4	gctggcaatgttcaacttcgtgtta	25	10192	10216	3	3CL pro	RNA	SARS-CoV infection
siSARS-5	gctctaagagccaactcagctgtta	25	12583	12607	4	Nsp8	RNA	SARS-CoV infection
siSARS-6	gcacatgtggctagttgtgatgcta	25	18768	18792	4	Nsp14	RNA	SARS-CoV infection

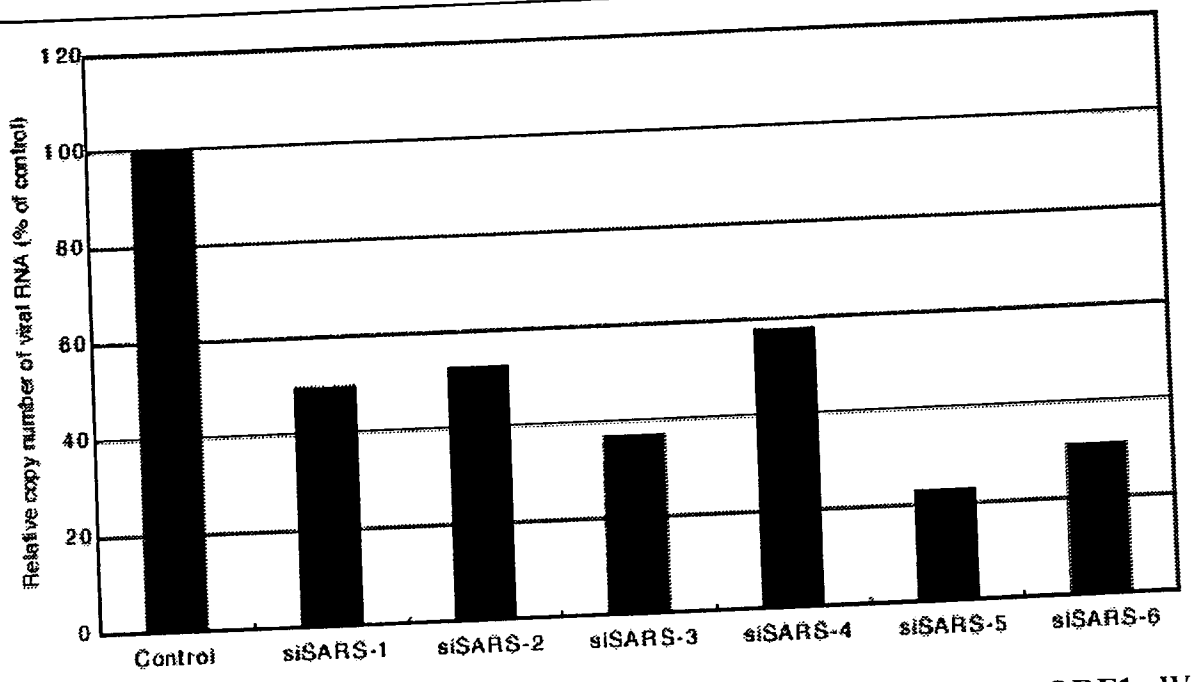

Figure 3. Inhibition of SARS-CoV replication by siRNAs targeting ORF1. We designed anti-SARS-CoV replicase siRNA with chemical modification and investigated the effect of siRNA on SARS-CoV replication by real-time RT-PCR. The targets of siSARS-1, -2, -3, -4, -5, and -6 were nsp 1, nsp 3, nsp3, 3CL proteinase, 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-expresson 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 administrated 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 accesory 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 retroviurs, 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 Tm value) and confers nuclease resistance. The 2'-O-methyl RNA is a natulally occuring 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 (IC50) 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 inhibiton 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 glycirrhizin, 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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NEW METHODS AND TECHNOLOGIES

Long-term transgene expression and inhibition of HIV-1 replication by a Cre/loxP-EBNA-1/oriP HIV-1-dependent ribozyme vector: Applications for HIV-1 gene therapy

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ABSTRACT

The cleavage of target mRNA by ribozymes is being exploited as a means of gene silencing in nucleic-acid-based therapies. We previously established an HIV-1-dependent ribozyme-expression vector system, based on Cre-loxP technology with an LTR-*gag-p17* promoter as a molecular switch for use in acute HIV-1 infection. The simultaneous expression of the Cre protein and loxP homologous recombination induced a high level of HIV-1-replication inhibition, but ribozyme expression was transient. In the current study, we overcame this limitation by inserting *EBNA-1* and *oriP* genes from the Epstein-Barr virus (EBV) into the vector. When this plasmid was introduced into HeLa CD4$^+$ cells, we observed long-term expression of both the *EGFP* reporter gene and the ribozyme. Moreover, HIV-1 replication was inhibited in the long-term in transfected cells. These data suggest that the HIV-1-dependent ribozyme-expression vector containing EBNA-1/oriP sequences would be a useful tool in HIV-1 gene therapy applications.

KEYWORDS: *Cre/loxP recombination, EBNA-1/oriP, gene therapy, HIV-1, ribozyme*

INTRODUCTION

In the application of therapeutics to HIV-1 infection, prevention is of greater importance than treatment. With this aim, many antiviral RNA-expression vector systems have been developed for use in anti-HIV-1 gene therapy (Banerjea et al, 2003; Barnor et al, 2004; Boden et al, 2004; Cordelier P et al, 2004; Habu et al, 2005; Mautino and Morgan, 2002; Takaku, 2004). Recently, small RNA molecules such as, siRNAs and snRNAs, have also been shown to affect gene silencing by RNA interference (Boden et al, 2003; Chang et al, 2002; Li et al, 2003).

Increasing the efficiency of transgene expression is of prime importance for human gene therapy (Davis and McNeilly, 2001). The Epstein-Barr Virus (EBV) is an autonomously replicating episomal vector that has been utilized to overcome the problem of rapid elimination of intracellularly delivered plasmid DNA in nonviral gene transfer. EBV is a gammaherpesvirus that is maintained as an episome of approximately 172 kb in size in a small number of resting B cells and epithelial cells in most of the human population. It latently infects human B cells with a high efficiency, after which its linear double-stranded genome circularizes and is sustained as a stable

episome (Hirai and Shirakata, 2001). The EBV replication system is present at a frequency of about 1-100 copies per cell, and maintains a non-covalent attachment to the host chromosome.

The latent origin of replication *oriP* and the viral transactivator protein EBNA-1 are essential components for EBV latent replication and maintenance of the viral genome (Daikoku et al, 2004; Lee et al, 1999). Both elements have been employed for long-term transgene expression in gene-therapy studies (Otomo et al, 2001; Tsujie et al, 2001).

Previously, we described an HIV-1-dependent ribozyme-expression vector capable of achieving site-specific excision of loxP sequences by using the HIV-1 minimal LTR-Cre-loxP system as a molecular switch in an acute HIV-1 infection (Habu et al, in press). However, we were unable to detect long-term expression of the anti-HIV-1 ribozyme. We hypothesized that the length of HIV-1-dependent transgene expression could be significantly increased in mammalian cells by introducing EBNA-1/oriP sequences to the vector.

In this study, we constructed an HIV-1-dependent long-term transgene (RNA ribozyme) expression vector (LTR-*gag-p17*/Cre-loxP-Rz-U5-*EBNA-1-oriP*-EGFP (EOG)) using the EBV replicon system, which was propagated in *Escherichia coli* and transfected into mammalian cells. We measured transgene-expression levels, including *EBNA-1* and *oriP*, in the presence and absence of HIV-1 infectious molecular clone (pNL4-3, Adachi et al, 1986). The potential anti-HIV-1 activity of the expression vector was evaluated with a view to establishing a highly effective therapeutic agent that could be further developed for HIV gene-therapy applications.

MATERIALS AND METHODS

Construction of plasmids

The retroviral vector pLEGFP-C1 (Clontech, Mountain View, CA) was digested with *Nhe* I and *Xho* I to release the DNA fragment encoding enhanced green fluorescent protein (EGFP). This was inserted into the *Nhe* I/*Xho* I sites of pCEP4 (Invitrogen, Carlsbad, CA), which contains *EBNA-1* and *oriP*, to create pCEP4-EGFP. An *Ssp* I fragment containing *EGFP*, *EBNA-1*, and *oriP* was cloned into the *Stu* I sites of pLTR-*gag-p17*-Cre/loxP-Rz-U5 (Habu et al, in press) and ploxP-Rz-U5, which been previously described with a high cleavage affinity (Habu et al, 2002) to yield pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG (Figure 1A) and ploxP-Rz-U5-EOG (Figure 1C), respectively. The control plasmid vector ploxP-Rz-U5-EOG lacks the LTR-*gag-p17*-Cre gene and so does not trigger expression of the ribozyme. A *Pvu* II fragment containing the EGFP-expression unit was excised from pCMV-EGFP previously constructed (unpublished data) and cloned into the *Stu* I sites of ploxP-Rz-U5 (Habu et al, 2002) or pLTR-*gag-p17*-Cre/loxP-Rz-U5 to generate ploxP-Rz-U5-G (Figure 1D) and pLTR-*gag-p17*-Cre/loxP-Rz-U5-G (Figure 1B), which are the EBNA-1 and oriP negative-control plasmids, respectively.

Cell culture and transfections

HeLa $CD4^+$ cells were grown in RPMI 1640 medium (Sigma, Saint Louis, MO) supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. HEK 293T cells were grown in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. All cells were maintained at 37 °C in a 5% CO_2 atmosphere. HeLa $CD4^+$ and 293T cell transfections were carried out using FuGENE™6 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol.

Luciferase assay

Luciferase activity was measured with the PicaGene kit (Toyo-inki, Tokyo, Japan) according to the manufacturer's protocol. The target gene-expressing plasmid pNL4-3-luc (Akkina et al, 1996), lacking an *env* gene and with a firefly luciferase gene replacing the *nef* gene, was co-transfected into HeLa $CD4^+$ cells with the pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG plasmid, which expresses the ribozyme following Cre/loxP homologous recombination. Transfected HeLa $CD4^+$ cells were lysed in 200 µl PicaGene cell lysis buffer for 15 min and then harvested. Cell debris was removed by centrifugation. Centrifuged lysate (10 µl) was added to 100 µl luminous substrate, and the luminescent signal was immediately quantitated with a luminometer (Lumat LB 9507; Berthold, Bad Wildbad, Germany).

Flow cytometry

Transfected HeLa $CD4^+$ cells were trypsinized, washed twice in PBS, and fixed in PBS containing 1% formaldehyde. Direct fluorescence of EGFP was analyzed by FACS Calibur (Clontech). Data acquisition and analysis were performed with CellQuest software (Clontech). Gates for detection of EGFP were established using mock-transfected cells as background.

Fluorescent microscopy

To evaluate the self-replicating function of the loxP ribozyme as an index for stable transgene expression in cells, transfected HeLa $CD4^+$ cells were trypsinized and seeded at a low cell density. Direct fluorescence microscopy of EGFP was carried out at the mitotic stage of cell division, after each passage on days 1, 3, 7, 13, 19 and 25 and the data were acquired with a DP12 digital microscope camera (Olympus Company, Tokyo, Japan).

RNA isolation and RT-PCR

Total cellular RNA was isolated from transfected HeLa $CD4^+$ cells with the GenElute Mammalian Total RNA kit (Sigma) using the manufacturer's protocol. RNA samples were treated with DNase I (Promega, Madison, WI) according to the manufacturer's instructions. RT-PCR assays were carried out using previously described primers (Habu et al, 2002) and the RT-PCR high-Plus-kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol.

Assay of HIV-1 replication

HIV-1 production was monitored by determining the HIV-1 p24 antigen concentration. The culture medium from HeLa $CD4^+$ cells co-transfected with pNL4-3 and pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG was harvested on days 1,

3, 5, 7, 9, 11, 13 and 15 post-transfection. p24 Gag protein production was detected by the HIV-1 p24 CLEIA assay (Lumipulse, Fujirebio Inc., Tokyo, Japan), according to the manufacturer's protocol (Sakai et al, 1999).

RESULTS AND DISCUSSION

Design and construction of an HIV-1-dependent Cre-expression vector

Tissue-specific gene transfer and expression are crucial for the development of safe and effective gene-therapy protocols. To this end, the HIV-1 LTR can serve as an efficient and inducible promoter dependent on the HIV-1 trans-activation factor, Tat. In a previous study, we constructed the HIV-1-dependent RNA ribozyme expression Cre/loxP vector, pLTR-*gag-p17*-Cre/loxP-Rz-U5-G, which targets mRNAs encoded by the U5 region (548–578) of the LTR (Figure 1E). This vector showed HIV-1-dependent ribozyme expression in HeLa CD4$^+$ cells (Habu et al, 2002; Habu et al, in press), but expression was not long term. Hence, in the current study, we constructed an HIV-1-dependent expression vector containing Cre/loxP and EBNA-1/oriP sequences, with the aim of increasing the duration of transgene expression. Moreover, we inserted the reporter gene *EGFP* to enable visualization of transgene expression (Figure 1A). The plasmid vectors pLTR-*gag-p17*-Cre/loxP-Rz-U5-G (Figure 1B), ploxP-Rz-U5-EOG (Figure 1C), and ploxP-Rz-U5-G (Figure 1D) served as controls. The advantage of this vector system over previously reported ribozyme vector systems (Chang et al, 2002; Li et al, 2003) is that it is not constitutively expressed to trigger off non-specific inhibition, but specifically expressed only in the event of HIV infection.

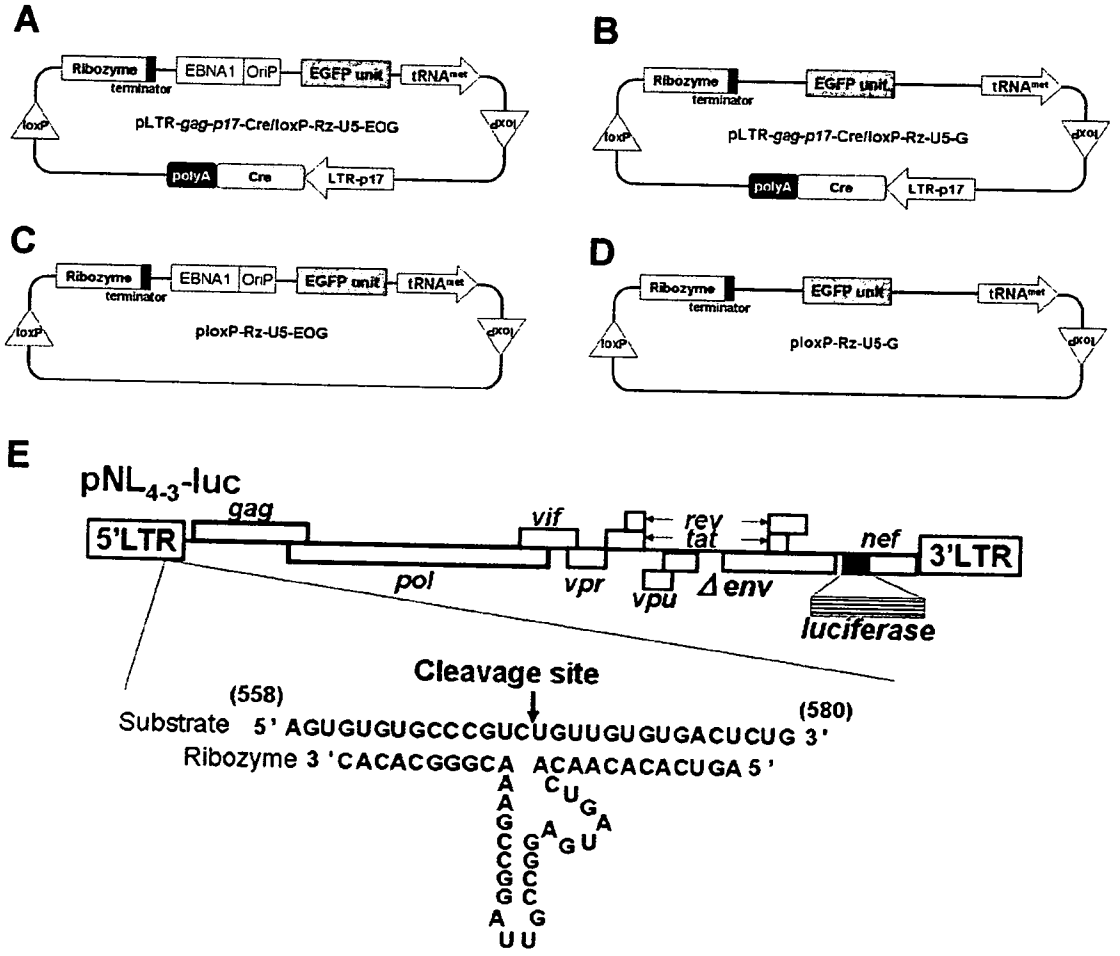

Figure 1. Schematic representation of HIV-1-dependent ribozyme-expression vectors. (A) The ribozyme expression (off switch) vector pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG, containing *EBNA-1* and *oriP* genes for long-term expression. (B) Control ribozyme-expression vector pLTR-*gag-p17*-Cre/loxP-Rz-U5-G, which lacks *EBNA-1* and *oriP* genes. (C) Control vector ploxP-Rz-U5-EOG, which lacks the *LTR-gag-p17-Cre* gene. (D) Control vector ploxP-Rz-U5-G, which lacks *EBNA-1/oriP* and *LTR-gag-p17-Cre* genes. (E) HIV-1 NL4-3 molecular clone pNL$_{4-3}$-luc containing the luciferase reporter gene, showing the target site and structure of the constructed ribozyme.

Long-term transgene expression of pLTR-gag-p17-Cre/loxP-Rz-U5-EOG in 293T and HeLa CD4$^+$ cells

To characterize the ability of the EBV replication system to effect long-term transgene expression we compared EGFP expression of control vector (pLTR-*gag-p17*-Cre/loxP-Rz-U5-G) with that of the EBNA-1/oriP plasmid pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG. EGFP expression in pLTR-*gag-p17*-Cre/loxP-Rz-U5-G-transfected 293T cells was observed for a maximum of 3 days, while EGFP expression persisted for more than 25 days in pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG-transfected 293T cells (Figure 2). As it has been reported that the EBNA-1 protein potentiates gene transcription (Mackey and Sugden, 1999), we measured the enhancement of gene expression by determining EGFP fluorescence intensity. FACS analysis showed that HeLa CD4$^+$ cells transfected with the EBNA-1-containing plasmids demonstrated longer-term EGFP expression (until day 7; Figure 3) than HeLa CD4$^+$ cells transfected with plasmids lacking EBNA-1. This is of therapeutic importance, because EBNA retains the therapeutic molecule to sensor for infective HIV-1 to release the catalytic ribozyme for cleavage.

Measurement of the mean fluorescence intensity (MFI) revealed a twofold increase in the EGFP expression level of HeLa CD4$^+$ cells transfected with pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG compared with pLTR-*gag-p17*-Cre/loxP-Rz-U5-G-transfected cells (data not shown). These results indicate that EBNA-1/oriP sequences mediate efficient and stable replication of transgene expression by enhancing nuclear localization of EBNA-1 (Mackey and Sugden, 1999; Marechal et al, 1999). The nuclear localization of this vector system is of cardinal importance since its function is induced by HIV-1 tat which is nuclear-based.

Dose-dependent inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG

The effect of pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG on HIV-1 (pNL_{4-3}-luc) replication was measured in a transient assay following its co-transfection with pNL_{4-3}-luc into HeLa CD4$^+$ cells. At 72 h post-transfection, the luciferase activity of cell lysate was measured as an indirect marker of viral replication. The plasmid vector ploxP-Rz-U5-EOG (Figure 1C), which does not trigger ribozyme expression as it lacks the LTR-*gag-p17*-Cre gene, was used as a control. Our analysis showed a dose-dependent inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG with a maximum inhibitory efficacy of >90% at a vector DNA concentration of 1.67 µg (Figure 4). Control vector alone had no inhibitory effect. This result suggests that the Rz-U5 ribozyme was expressed using Cre/loxP recombination and EBNA/oriP systems in HIV-1 infected cells, and successfully cleaved its target HIV-1 mRNA (Figure 1E).

EBNA-1 mediates long-term ribozyme expression in HIV-1-dependent vector-transfected HeLa CD4$^+$ cells

As long-term ribozyme-U5 (Rz-U5) expression is an important determinant of its efficiency, we compared the level of expression in HeLa CD4$^+$ cells transfected with pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG or pLTR-*gag-p17*-Cre/loxP-Rz-U5-G in the presence or absence of pNL4-3 (Figure 5). RT-PCR analysis showed that Rz-U5 expression persisted for more than 19 days following pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG transfection in the absence of pNL4-3 (Figure 5). By contrast, pLTR-*gag-p17*-Cre/loxP-Rz-U5-G-transfected cells demonstrated Rz-U5 expression for only 3 days. Ribozyme expression was not observed following transfection of either plasmid DNA in the absence of pNL4-3. These results confirm that

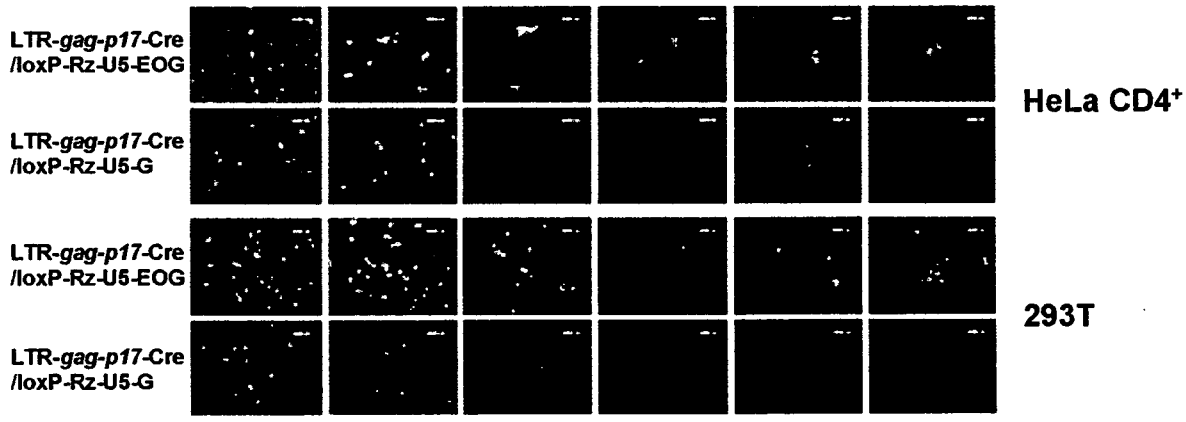

Figure 2. *EBNA-1* and *oriP* increase the duration of EGFP expression in transfected HeLa CD4$^+$ and 293T cells. EGFP expression was evaluated on days 1, 3, 7, 13, 19, and 25 post-transfection.