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RNA Interference Targeted to the Conserved Dimerization Initiation Site (DIS) of HIV-1 Restricts Virus Escape Mutation

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Short hairpin RNAs (shRNA) targeting viral or cellular genes can effectively inhibit human immunodeficiency virus type 1 (HIV-1) replication. This inhibition, however, may induce mutations in the targeted gene, leading to rapid escape from the shRNA-induced inhibition. We generated a lymphoid cell line that stably expressed a 19-bp shRNA targeting a well-conserved dimerization initiation site (DIS) of HIV-1, which strongly inhibited viral replication, thereby delaying virus escape. Furthermore, treatment of HIV-1 infection with DIS- and vif-shRNA combination therapy resulted in superior anti-viral responses compared to vif-shRNA monotherapy. Continuous challenge with HIV-1, however, generated virus mutants that could overcome the RNA interference restriction. Such anti-genes may be promising tools for HIV-1 gene therapy for HIV/acquired immunodeficiency syndrome.

Key words: combination therapy, lentiviral vector, HIV-1, RNA interference, well-conserved dimerization initiation site (DIS), virus escape mutation.

Abbreviations: HIV-1, human immunodeficiency virus type1; DIS, dimerization initiation site; vif, HIV-1 virion infectivity factor; RNAi, RNA interference; shRNA, short hairpin RNA; EGFP, enhanced green fluorescent protein.

RNA interference (RNAi) is a natural biological phenomenon mediated by small interfering RNA (siRNA) molecules that target specific mRNA for degradation by cellular enzymes. RNAi has become a popular method for studying gene function, especially in mammalian systems. With proof-of-concept studies already presented against a diverse range of human pathogens and several innovative methods described for delivering siRNA to a wide variety of primary cells, there is great potential for siRNA as a therapeutic strategy (*1–8*). Many groups have reported the general use of siRNAs to specifically inhibit HIV-1 replication by targeting viral or cellular genes (*9–17*), suggesting a role for RNAi in the therapeutic treatment of HIV-1 infection.

One disadvantage of targeting viral genes with RNAi, however, is that viral mutations can lead to a loss of sensitivity to RNAi. This is particularly problematic in the case of RNA viruses, which accumulate point mutations up to 107 times more rapidly than DNA viruses (*18*). RNAi-resistant mutants have been shown to arise in cell culture models while targeting HIV (*19–22*), poliovirus (*23*, *24*), hepatitis C virus (*25*) and hepatitis B virus (*26*). Although most of these mutants achieved resistance through a point mutation or deletion of the target sequence, one HIV mutant escaped RNAi suppression by accumulating mutations outside of the target sequence, thereby creating a new local RNA secondary structure that presumably excluded the RNA-induced silencing complex (*21*). These findings demonstrate that any effective RNAi-based anti-viral therapy must compensate for the evolutionary potential of the pathogen. Importantly, work with HIV-1 also indicates that tolerance to target sequence mismatches may depend on the particular sequence of the siRNA tested (*27*). Sabariegos *et al*. (*22*) reported that optimal HIV-1 gene silencing by siRNA requires complete homology within the central region of the target sequence, and that substitutions at only a few positions at the 5′ and 3′ ends are partially tolerated. Thus, targeting single sequences in the viral genome as a therapeutic approach for RNA viruses has limited efficacy.

In the present study, we found that siRNAs that specifically target the well-conserved dimerization initiation site (DIS) (*28–30*) of HIV-1, compared to those targeting non-conserved regions, drive RNAi-resistant mutants to emerge at a slower rate. We selected the HIV-1 vif region as a target site and constructed a lentiviral vector expressing the shRNA. The HIV-1 *vif* gene encodes a highly basic, 23,000-M_r phosphoprotein that collapses intermediate filaments, localizes in the cytoplasm of its infected target cells, and acts during virus assembly by an unknown mechanism to enhance viral infectivity (*31–33*). The siRNAs targeting the DIS drive the accumulation of point mutations at 77 days, almost 2 months slower than those targeting the vif region. Furthermore, coexpression of DIS- and vif-siRNAs

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following lentiviral-mediated transduction of SupT1 cells provides enhanced, longer-term inhibition of HIV-1 infection relative to single expression. The data support the utility of combining different anti-viral modalities in a gene therapy setting to effectively suppress HIV-1 replication.

MATERIAL AND METHODS

Construction of U6 Expression Plasmids and Lentiviral-based Vectors—A short hairpin RNA (shRNA) was designed to target the DIS and vif sequences (Fig. 1A). The shRNA sequences were chemically synthesized as two complementary DNA oligonucleotides: (DIS sense: 5′-CGGCTTGCTGAAGCGCGCACGGTTCAAGAG ACCGTGCGCGCTTCAGCAAGCCTTTTTTTCTAGAG-3′; DIS antisense: 5′-GATCCTCTAGAAAAAAAGGCTTGCT GAAGCGCGCACGGTCTCTTGAACCGTGCGCGCTTCA GCAAGCCGGTAC-3′; vif sense: 5′-CCAGATGGCAGGTG ATGATTGTTTCAAGAGAACAATCATCACCTGCCATCT GTTTTTTTCTAGAG-3′; vif antisense: 5′-GATCCTCTAG AAAAAAACAGATGGCAGGTGATGATTGTTCTCTTGAA ACAATCATCACCTGCCATCTGGGTAC-3′; LacZ sense: 5′-CGTGACCAGCGAATACCTGTTCTTCAAGAGAGAAC AGGTATTCGCTGGTCACTTTTTTTCTAGAG-3′; LacZ antisense: 5′-GATCCTCTAGAAAAAAAGTGACCAGCGA ATACCTGTTCTCTCTTGAAGAACAGGTATTCGCTGGT CACGGTAC-3′). The oligonucleotides were mixed in equimolar amounts, heated for 5 min at 95°C, and then gradually cooled to room temperature in annealing buffer (10 mM Tris–HCl, 100 mM NaCl). The resultant duplex was ethanol-precipitated and ligated into *Kpn*I and *Bam*HI cloning sites upstream of the U6 promoter of pSV2neo (TAKARA, Otsu, Japan). The U6 DIS-shRNA vector encoded HIV-1 DIS, the U6 LacZ shRNA vector encoded LacZ and the U6 vif-shRNA vector encoded vif.

To construct the lentiviral vectors, the *Eco*RI fragment of the U6 vectors listed above containing the U6 promoter and the siRNA duplex was cloned into the *Eco*RI site of the lentiviral transfer vector (pCS-CDF-CG-PRE), generating the CS-DIS-shRNA, CS-vif-shRNA, and control the transfer vectors CS-LacZ-shRNA and CS-U6-ter.

Cell Culture—Sup-T1 cells were grown in either RPMI 1640 (Sigma, St Louis, MO, USA) or Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). All cultures were maintained at 37°C under a 5% CO_2 atmosphere.

Lentivirus Preparation—293T cells were co-transfected with 15 μg transfer vector construct, 15 μg helper constructs coding for Gag-Pol (pMDLg/p.RRE), 5 μg Rev-expressing construct pRSV-Rev and 5 μg VSV-G-expressing construct pMD.G, using the calcium phosphate precipitation method (*34*). Supernatants were harvested 72 h post-transfection, filtered through a 0.45 μm filter disc and concentrated 100-fold by centrifugation at 6,000*g* overnight. The resultant viral pellet was resuspended in serum- and antibiotic-free RPMI medium and stored at –80°C until use. To determine the viral titre, 5×10^5 293T cells were transduced with the prepared viral stock, and the number of EGFP-positive cells was counted after a 72 h culture using flow cytometric analysis (*35*).

Transduction of SupT1 Cells—SupT1 cells (3×10^5) were seeded in 12-well plates in 1 ml culture medium. Cells were transduced with the CS-U6-shRNAs and control lentiviral vectors at a multiplicity of infection (MOI) of 20 in the presence of 4 μg/ml polybrene. After incubation at 37°C for 4 h, the cells were washed three times with phosphate buffered saline (PBS) and resuspended in growth medium.

HIV-1 Challenge and Long-term Culture Assay—After transduction, SupT1 cells expressing the transgenes were challenged with HIV-1_{NL4-3} at an MOI of 0.1. Following infection, the cells were washed three times with PBS and resuspended in growth medium. Mock infection was performed under the same conditions except that the supernatants were generated from control/vector-transduced cells. Half of the culture volume was harvested and replaced with an equal volume of fresh culture medium at regular intervals. The harvested

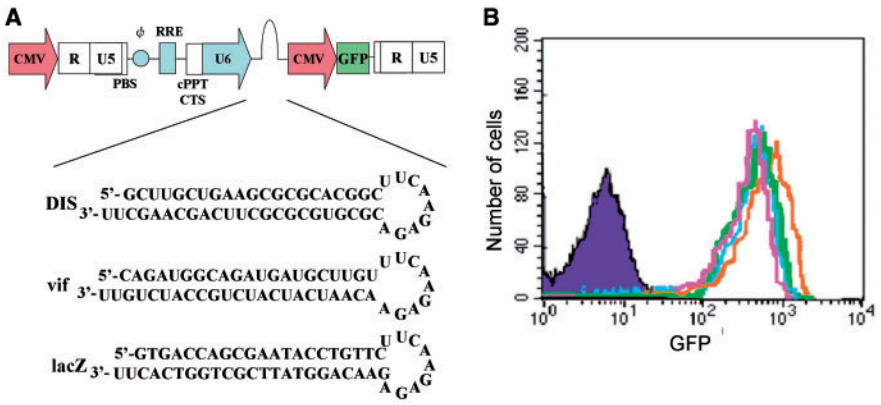

Fig. 1. **RNAi targets in the HIV-1 genome and the shRNA vector.** (A) Lentiviral vectors were designed to express shRNA against the following regions of the HIV-1 sequence: 246–266 nt of the DIS sequence, containing a stem–loop structure with six self-complementary nucleotides at the top; and 5049–5069 nt of the vif sequence. Expression of the shRNA was driven by the pol III. (B) The transgenic EGFP expression in SupT1 cells expressing vector transgenes was examined by FACS analysis using *CELLQUEST* software. Purple, (SupT1); green, CS-U6-DIS-shRNA; red, Cs-U6-vif-shRNA; blue, CS-U6-lacZ-shRNA; orange, CS-U6-ter.

culture was centrifuged and the cell-free medium was used for HIV-1 gag p24 antigen quantification and viral RNA extraction, while the pellet was used for cell-viability counts and FACS analysis of EGFP expression as a marker of transgene expression.

Flow Cytometry Analysis of Long-term EGFP Expression—Half of the culture volume was harvested at 7 and 28 days (vif-shRNA), and 63, 70 and 98 days (DIS-shRNA) post-challenge, pelleted, washed twice in PBS and resuspended in 1% formaldehyde. FACS analysis was performed using the FACS Calibur and *CELLQUEST* software (BD Sciences, San Jose, CA, USA).

Fluorescence Microscopy—Fluorescence microscopy was used to investigate the efficiency of EGFP expression as an index of transgene expression in the SupT1 cells. For intracellular fluorescence studies, SupT1 cells were fixed with 3.7% formaldehyde on alternate days. Fluorescence-activated cells were examined under fluorescence microscopy (Biozero BZ-8000; KEYENCE, Osaka, Japan) at an excitation wavelength of 488 nm using a 10× objective lens. Images were acquired at a 512×512 pixel resolution.

Generation of Viruses—To generate HIV-1 viruses, the HIV-1 infectious molecular cloned plasmid vector (HIV-1pNL4-3) was transfected with 3 μg DNA into 5×10^5 seeded HeLa $CD4^+$ cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The culture was incubated at 37°C for 72 h and harvested. The cells were then pelleted by centrifugation to produce a cell-free supernatant yielding the HIV-1_{NL4-3} virus, which was aliquoted and stored at −80°C. HIV-$1_{NL4-3\text{-}vif\text{-}mut\ (G\text{-}C)}$ virus was generated from the experiment illustrated in Fig. 4B. To summarize, SupT1 cells were transduced with lentivirus-mediated DIS- or vif-shRNAs at an MOI of 20 in the presence of 4 μg/ml polybrene. After incubation at 37°C for 4 h, the cells were washed three times with PBS and resuspended in growth medium. The cells were then challenged with HIV-1_{NL4-3} virus at an MOI of 0.1 and cultured for 98 d at 37°C. At 21 days, harvested supernatant showing a vif mutation virus, HIV-$1_{NL4-3\text{-}vif\text{-}mut\ (G\text{–}C)}$ (siRNA vif target 5049-CAGATG GCAGGTGATGATTGTGT-5569; 5049-CACATGGCAGG TGATGATTGTG-5569: 1 nt substitution), was titred and stored at −80°C until use.

Genotypic Sequence Analysis of the DIS- and vif-siRNA Target Regions of HIV-1_{NL4-3} and Re-challenge of Cells Expressing DIS- and vif-shRNA—Viral RNA from HIV-1_{NL4-3}-challenged CS-DIS or CS-vif shRNA-transduced cultures was analyzed for siRNA-mediated mutations in the vif-shRNA target region at weeks 2 through 14. Viral RNA was isolated from the cell-free culture supernatant using the QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Viral RNA (5 μl) was used in a reverse transcription–PCR containing Powerscript reverse transcriptase (Clontech, Mountain View, CA, USA), 1 mM each of the deoxynucleotide triphosphates, 1× first-strand buffer (Clontech), 200 ng random hexamer (Promega, Madison, WI, USA) and 10 U RNasin (Promega). Reverse transcription was performed at 42°C for 1 h, followed by heat inactivation of the reverse transcriptase enzyme at 70°C for 15 min. cDNA (2 μl) was added to a 48 μl PCR mixture containing 1× Qiagen Taq PCR buffer, 1.5 mM $MgCl_2$, 20 pmol sense primer DIS F (5′-GTGT GGAAAATCTCTAGCAGTGGGG-3′), antisense primer DIS R (5′-CCTCAATAAAGCTTGCCTTGAGTGCT C-3′), sense primer vif F (5′-CGGGTTTATTACAGGGACA GCAGAGA-3′), antisense primer vif R (5′-AAAGGTGAAG GGGCAGTAGTAATACAAGAT-3′), 1 mM each of the deoxynucleotide triphosphates, and 2.5 U Taq polymerase (Qiagen). PCR was performed using a gradient PCR thermal cycler (Astec, Fukuoka, Japan) with the following thermal program: 1 cycle (95°C for 1 min), 35 cycles (95°C for 15 s, 58°C for 30 s, 72°C for 30) and 1 cycle (72°C for 5 min). The PCR product was fractionated, analysed on a 1% SeaKem gel, and purified using a QIAEX II gel extraction kit (Qiagen). Nucleotide cycle sequencing was performed using dye-labeled terminator chemistry.

SupT1 cells stably expressing DIS-shRNA, vif-shRNA, LacZ-shRNA or the control U6-ter were challenged with either wild-type virus HIV-1_{NL4-3} or mutant virus HIV-$1_{NL4-3\text{-}vif\text{-}mut\ (G\text{–}C)}$. SupT1 cells ($1 \times 10^6$) were infected with 50 ng of gag p24 antigen from each virus. Following infection, the cells were washed three times with PBS and resuspended in growth medium. The time-course of the infection was monitored over an 8-day period by HIV-1 gag p24 enzyme-linked immunosorbent assay.

RESULTS

Long-term Inhibition of HIV-1 Replication by Lentiviral Vector-mediated DIS and vif-shRNA—Our major goal in these studies was to introduce both DIS- and vif-shRNAs into a lentiviral construct to achieve their stable expression in transduced cells. Lentiviral vectors offer advantages over conventional retroviral vector systems because they can transduce both dividing and non-dividing cells, and are less prone to transgene silencing (*36–38*). We previously reported the design of lentiviral vectors expressing shRNA against the following regions of HIV-1: 246–266 nt of the DIS region, containing a stem–loop structure with six self-complementary nucleotides at the top (*39*) and 5049–5069 nt of the vif region (*40*).

The transfer vector pCS-U6-shRNA-EGFP contained short-hairpin type DIS, vif, and LacZ-siRNAs driven by a Pol-III U6 promoter. Downstream, the reporter gene EGFP was driven by a cytomegalovirus promoter. The plasmid vector, pCS-U6-ter-EGFP, lacked a short hairpin siRNA (Fig. 1A).

SupT1 cells transduced with the control vectors CS-U6-LacZ-shRNA and CS-U6-ter, or the CS-U6-DIS- and vif-shRNA vectors, showed 98%, 99%, 97% and 92% EGFP expression, respectively, as measured by FACS analysis. This indicates high transduction efficiency (Fig. 1B).

To determine whether HIV-1 was down-regulated by the shRNA in the DIS-shRNA construct, the transduced cells were tested for HIV-1 gag p24 antigen. At 70 days, the HIV-1 gag p24 antigen levels were significantly reduced in DIS-shRNA transduced cells compared to cells transduced with the control vectors

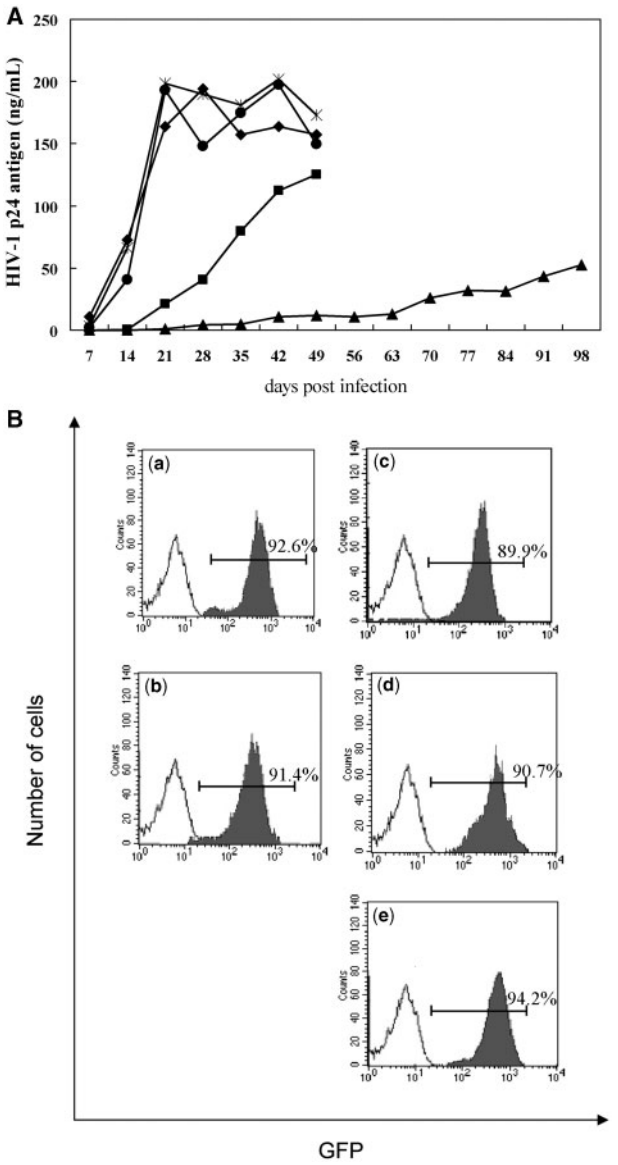

Fig. 2. **Long-term activity of lentivirus-mediated DIS- and vif-shRNA.** (A) Long-term inhibition of HIV-1 replication in SupT1 cells. HIV-1 gag p24 antigen was measured during the 98-day culture of SupT1 cells transduced with the indicated CS-lentiviruses (MOI 20), and challenged with HIV-1_{NL4-3} (MOI 0.1). Negative control, asterisk; vif-shRNA, solid square; LacZ-shRNA, solid diamond; DIS-shRNA, solid triangle; U6-ter, solid circle. (B) Long-term expression of transgenic EGFP in Sup-T1 cells expressing vector transgenes was examined by FACS analysis using *CELLQUEST* software. a, vif-shRNA at 7 days; b, vif-shRNA at 28 days; c, DIS-shRNA at 63 days; d, DIS-shRNA at 70 days; e, DIS-shRNA at 98 days.

CS-U6-LacZ-shRNA and CS-U6-ter (Fig. 2A). Furthermore, SupT1 cells transduced with the CS-U6-DIS-shRNA vector showed ~94% EGFP expression at 98 days (Fig. 2B-e). A steady increase in viral expression was observed, however, after day 70 (Fig. 2A). The anti-HIV-1 activity of vif-shRNA was similarly evaluated. Viral expression steadily increased from 21 days post-infection in the transduced SupT1 cells (Fig. 2A), whereas EGFP reached 91% expression at 28 days (Fig. 2B-b). These results demonstrate that DIS-shRNA results in the generation of resistant viruses 70 days later than vif-shRNA.

RNAi-resistant HIV-1 Variants—Long-term cultures stably expressing siRNAs targeting HIV genes eventually give rise to escape mutants (*20–22*). We therefore investigated the sudden increase in viral replication in cultures expressing vif- and DIS-shRNAs. Sequence analyses showed resistance against their respective shRNAs (Fig. 3A and B). DIS-shRNA showed a potential inhibition of HIV-1 replication compared with vif-shRNA at 77 days post-infection. Most surprising was the emergence of RNAi-resistant viruses containing just a single nucleotide substitution (A256T) on the DIS-shRNA target sequence (Fig. 3A). Notably, hyper-reactivity of the last adenine in the DIS loop (A256), which is the hallmark of *in vitro* RNA dimerization (*41*, *42*), was observed in infected cells (*43*). We also observed the emergence of RNAi-escape viruses with resistance against vif-shRNA that contained nucleotide substitutions or deletions in or near the vif-shRNA target sequence (Fig. 3B).

To determine the inhibitory effects of vif-shRNA and DIS-shRNA on HIV-$1_{NL4-3-vif-mut\ (G–C)}$ and wild-type HIV-1_{NL4-3}, SupT1 cells stably expressing either vif-shRNA or DIS-shRNA were infected with HIV-$1_{NL4-3-vif-mut\ (G–C)}$ and HIV-1_{NL4-3}. When control cells were infected with the wild-type virus and escape virus, both viruses showed a similar replication curve (Fig. 4A). HIV-1_{NL4-3} and HIV-$1_{NL4-3-vif-mut\ (G–C)}$ both caused a potential inhibition of HIV-1 replication at 8 days in SupT1 cells expressing the DIS-shRNA (Fig. 4B). In SupT1 cells expressing vif-shRNA, the RNAi-resistant virus (HIV-$1_{NL4-3-vif-mut\ (G–C)}$) replicated at the same rate as in control cells (Fig. 4C). These results demonstrate that the efficiency of siRNA binding to target RNA can be diminished by nucleotide substitutions or deletions in the target sequence.

Multi-targeting with both DIS- and vif-shRNAs Results in Long Lasting Inhibition of HIV-1 Genes—To test the long-term ability of vif- and DIS-shRNAs to inhibit target gene expression in SupT1 cells, the cells were transduced with either CS-U6-vif-shRNA alone, or with a combination of CS-U6-vif and DIS-shRNAs. In those cells transduced with CS-U6-vif-shRNA alone, a potential inhibition of HIV-1 replication was observed 14 days post-infection. This inhibition was gradually reduced to 65% by 34 days. Other vif-shRNA-expressing cells challenged with HIV-1_{NL-4-3} were further challenged with CS-U6-DIS-shRNA at 21 days. The HIV-1 gag p24 antigen levels were significantly reduced in CS-U6-vif-shRNA/CS-U6-DIS-shRNA transduced cells compared to cells transduced with CS-U6-vif-shRNA alone for 34 days (Fig. 5A). SupT1 cells transduced with CS-U6-vif-shRNA vectors, however, expressed EGFP at 28 days (Fig. 5B-b).

DISCUSSION

In contrast to acute viral infections, chronic infections with viruses such as HIV-1, hepatitis C virus and herpes simplex virus should be targeted with long-term RNAi treatment. In the case of HIV-1, virus-resistant

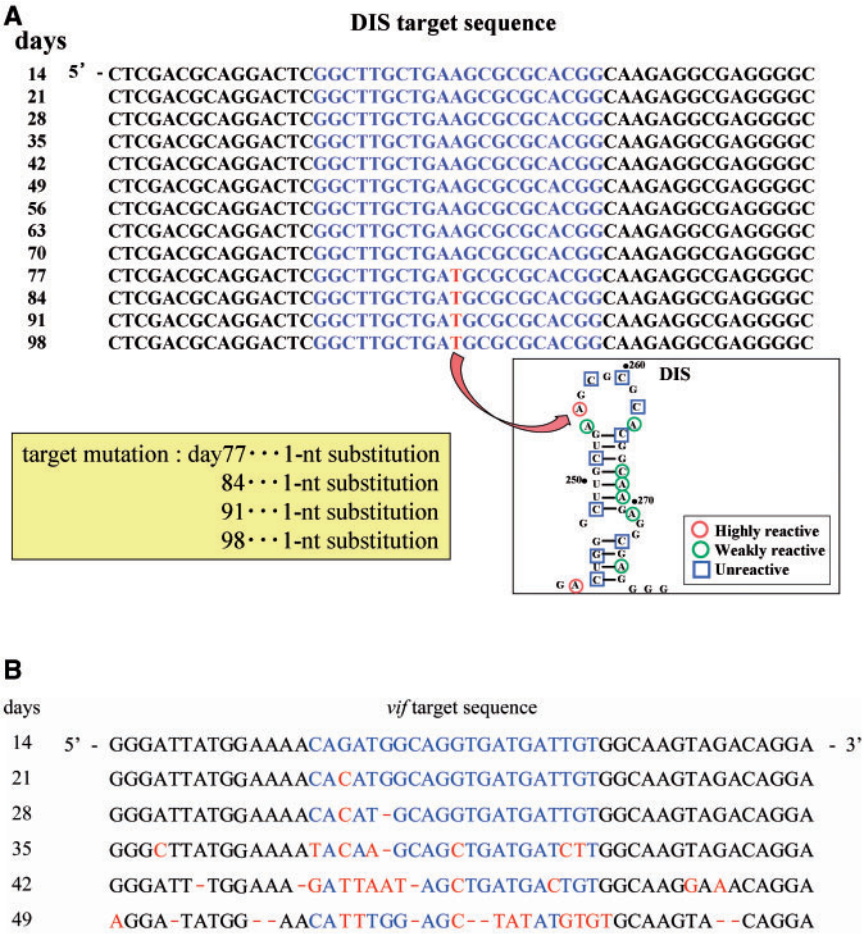

Fig. 3. **HIV sequence variation following shRNA-escape.** (A) Genotype sequence analysis revealed siRNA-mediated mutations in the DIS-shRNA target site (nucleotides 246–266; blue) of HIV-1_{NL4-3} in RNA extracted from DIS-shRNA-expressing culture supernatants. Day of sequencing is indicated. Substitutions are indicated in red. (B) Genotype sequence analysis revealed siRNA-mediated mutations at the vif-shRNA target site (nucleotides 5049–5069, blue) of HIV-1_{NL4-3} in RNA extracted from vif-shRNA-expressing culture supernatants. Day of sequencing is indicated. Deletions are indicated by dashes; substitutions are indicated in red.

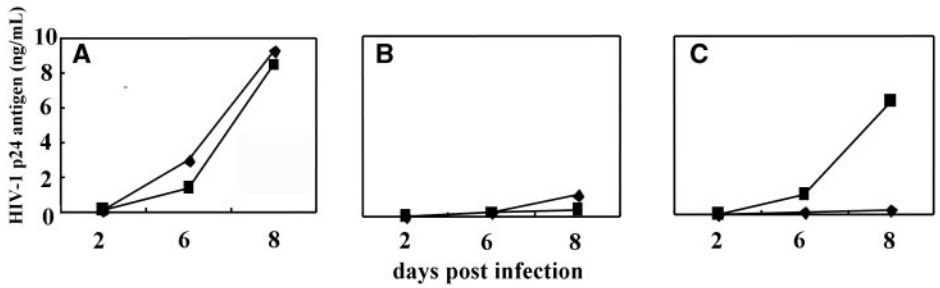

Fig. 4. **Inhibitory effects of vif-shRNA and DIS-shRNA on wild-type or vif-shRNA resistant virus (HIV-$1_{NL4-3\text{-}vif\text{-}mut\ (G–C)}$).** (A) Control cells were infected with the vif mutant virus HIV-$1_{NL4-3\text{-}vif\text{-}mut\ (G–C)}$ (solid square) and the wild-type HIV-1_{NL4-3} virus (solid diamond). (B) DIS-shRNA cells were infected with HIV-$1_{NL4-3\text{-}vif\text{-}mut\ (G–C)}$ (solid square) and HIV-1_{NL4-3} virus (solid diamond). The vif mutant virus HIV-$1_{NL4-3\text{-}vif\text{-}mut\ (G–C)}$ was strongly inhibited by DIS-shRNA (solid square). (C) vif-shRNA cells were infected with HIV-$1_{NL4-3\text{-}vif\text{-}mut\ (G–C)}$ (solid square) and HIV-1_{NL4-3} virus (solid diamond). The vif mutant virus HIV-$1_{NL4-3\text{-}vif\text{-}mut\ (G–C)}$ was resistant to DIS-shRNA (solid square). Replication was monitored by measuring levels of the p24 antigen up to 8 days post-infection.

$CD4^+$ T cells can be produced by *ex vivo* transduction of blood stem cells to express the anti-HIV-1 RNAi trigger before re-introducing these cells to the patient (*44*). Lentiviral vectors are used to stably transduce cells with shRNA expression constructs, resulting in strong inhibition of virus replication (*37*, *38*). Prolonged culturing of these cells, however, results in the selection of escape variants resistant to the expressed siRNA; thus,

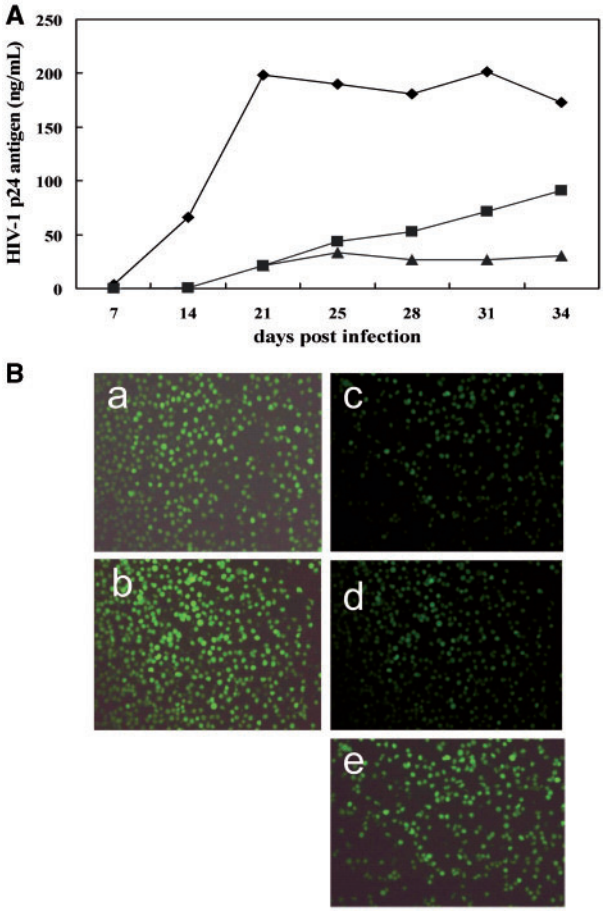

Fig. 5. **Multi-targeting with both DIS- and vif-shRNAs results in longer-lasting inhibition of HIV-1 genes.** (A) vif-shRNA-expressing cells were challenged with HIV-1_{NL-4-3} and the cells were cultured. On day 21, the cells were further challenged with CS-U6-DIS. The levels of HIV-1 gag p24 antigen were reduced significantly in DIS-shRNA-expressing cells compared to vif-shRNA cells alone for 34 days. vif-shRNA, solid square; negative control, solid diamond; vif-shRNA/DIS-shRNA, solid triangle. (B) Expression of transgenic EGFP in Sup-T1 cells expressing vector transgenes was examined by fluorescence microscopy. a, vif-shRNA at 7 days; b, vif-shRNA at 21 days; c, vif-shRNA/DIS-shRNA at 21 days; d, vif-shRNA/DIS-shRNA at 28 days; e, vif-shRNA/DIS-shRNA at 34 days.

the RNAi-induced block of HIV-1 replication is not durable. Nucleotide substitutions or deletions within the siRNA-target sequence of resistant variants may affect important regulatory sequences and/or essential amino acids, resulting in reduced viral fitness (*19*, *20*).

In this study, we described a slower rate of emergence of RNAi-resistant mutants by targeting siRNA to the well-conserved DIS of HIV-1 (*28–30*). The DIS is a stem–loop structure with six self-complementary nucleotides at the top, located between the primer binding site and the splice donor site at the end of a long terminal repeat (*45*). The DIS is involved in the dimerization of the HIV-1 genome, packaging and proviral synthesis (*30*, *46–48*). There are two major motifs in HIV-1: GUGC AC in subtypes A and C, and GCGCGC in subtypes B and D (*49*, *50*). The subtype A motif is a good target for siRNA in an *in vitro* cell-free system where HIV-1 genome dimerization is successfully inhibited by a 9-mer DIS-targeting siRNA, while the corresponding DNA oligonucleotide does not affect dimerization (*51*, *52*).

To determine whether HIV-1 is down-regulated by the expressed shRNA in the CS-U6-DIS-shRNA construct, the transduced cells were tested for HIV-1 gag p24 antigen expression. During the first 70 days post-infection, HIV-1 replication was significantly reduced in CS-U6-DIS-shRNA transduced cells (Fig. 2A). By 77 days, however, p24 antigen levels had increased, indicating the loss of the DIS-shRNA-mediated anti-viral activity (Fig. 2A). Flow cytometry analysis revealed continued intracellular production of the reporter gene EGFP (Fig. 2B). Similarly, viral expression dramatically decreased in CS-U6-vif-shRNA transduced cells for 14 days, followed by an increase beginning at 21 days (Fig. 2A). DIS-shRNA expression in the cells results in the generation of escape mutants 70 days slower than vif-shRNA.

To determine whether HIV-1 escape from RNAi was due to the emergence of mutations within the DIS and *vif* genes, viral RNA was extracted from sequential samples of culture supernatant and the DIS and vif-shRNA target regions were amplified by PCR and sequenced. Any single-nucleotide mismatch between the siRNA and the DIS target sequence is capable of reducing the silencing effect. In particular, a single nucleotide change at the center of the target sequence (A256T), with the exception of position 11, was very effective in allowing the virus to escape siRNA inhibition (Fig. 3A). Notably, hyper-reactivity of the last adenine in the DIS loop (A256), which is the hallmark of *in vitro* RNA dimerization (*41*, *42*), was observed in infected cells (*43*). More recently, Jones *et al*. (*53*) reported that the mutations in a non-coding region of the HIV-1 RNA genome affect the ability of the virus to synthesize viral cDNA in a cell type-dependent manner, illustrating the importance of virus–host cell interactions via an RNA-trigger. Most surprising, there was an emergence of RNAi-resistant viruses that contained nucleotide substitutions or deletions in or near the vif-shRNA target sequence (Fig. 3B). Other studies have suggested that nucleotide polymorphisms at the 3′- and 5′-end of the target sequence do not strongly affect recognition by siRNA (*21*, *54*). Furthermore, Du *et al*. (*55*) reported that target sequences containing mutations at position 12 are well tolerated by the silencing machinery. In addition, HIV-1 silencing is affected by a nucleotide change at residue 12 in a *gag* siRNA target sequence (*27*). Our results show that the efficacy of HIV-1 silencing can be reduced by a single-nucleotide polymorphism at the target sites (Fig. 3A). We also found that DIS-shRNA-expressing cells can slow the generation of escape mutants by about 2 months compared with vif-shRNA.

Lee *et al*. (*27*) reported that targeting conserved sequences, such as the *vif* sequence of HIV-1, delays the emergence of virus escape mutants. We, however, observed nucleotide substitutions or deletions in or near the vif sequence targeted by vif-shRNA. These discrepancies highlight the possible relevance of a specific target sequence in siRNA recognition and escape

from RNAi. Furthermore, not all siRNAs are equally effective, and it is difficult to predict the potential value of a particular siRNA (*56*).

To test the long-term ability of DIS- and vif-shRNAs to inhibit target gene expression, cells were transduced with either vif-shRNAs alone or with a combination of DIS- and vif-shRNAs, and challenged with wild-type HIV-1_{NL-4-3}. In the vif-shRNA transduced SupT1 cells, an increase in viral expression was observed from 21 days (Fig. 5A). Other vif-shRNA-expressing cells challenged with HIV-1_{NL-4-3} were further challenged with CS-U6-DIS-shRNA beginning at day 21. The levels of HIV-1 gag p24 antigen were reduced significantly in these cells compared to the cells transduced with CS-U6-vif-shRNA alone for 34 days (Fig. 5A). These results suggest that HIV-1 may escape the anti-viral activity of RNAi by specific mutations in the targeted sequence, and that targeting conserved sequences and the simultaneous use of multiple siRNAs may be useful strategies in maintaining inhibition of viral replication. Second generation siRNAs that recognize the mutated target sites may be used to prevent the emergence of resistant virus (*54*).

In conclusion, we highlighted specific target loci within the 5′ long-terminal repeat of HIV-1 that are susceptible to shRNA targeting, and may prove advantageous over other RNAi target sites within HIV-1. Although shRNAs require further manipulation to improve their overall efficacy in generating multiple functioning siRNAs, they may prove to be a useful tool in the multifaceted approach to treating HIV-1 infection. In fact, RNAi might be combined effectively with any number of anti-viral therapeutics, including dominant negative antiviral proteins. Future work is needed to develop these principles to identify anti-viral strategies that provide effective and long-term suppression of viral replication and pathogenesis.

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CONFLICT OF INTEREST

None declared.

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