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Inhibition of HIV-1 replication by long-term treatment with a chimeric RNA containing shRNA and TAR decoy RNA

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ABSTRACT

Combinatorial therapies for the treatment of HIV-1 infection are effective for reducing patient viral loads and slowing the progression to AIDS. Our strategy was based on an anti-HIV-1 shRNA vector system in which HIV-1 vif-shRNA was fused to a decoy TAR RNA (mini-TAR RNA) to generate vif-shRNA-decoy TAR RNA under the control of the human U6 Pol III promoter. Upon expression in human cells, the RNA molecule was cleaved into its component parts, which inhibited HIV-1 replication in a synergistic manner. This chimeric RNA expressed a dual RNA moiety and greatly enhanced the inhibition of HIV-1 replication under the production of resistant virus by short interference RNA (siRNA) in long-term culture assays. We suggest that this technique provides a practical basis for the application of siRNA-based gene therapy in the treatment of HIV/AIDS.

1. Introduction

The emergence of multiple drug-resistant strains of HIV-1 has necessitated the development of alternative approaches for AIDS treatment (Cogoni et al., 1994). Unfortunately, chemotherapy adjuvants such as gene therapy are hampered by difficulties in delivering therapeutic genes to target cells. Lentiviral vectors, however, can be used for long-term and sustained gene expression and have the additional advantage of not requiring cell division to enter the cell nucleus.

RNA interference (RNAi) is a powerful tool for suppressing gene function and has generated much excitement in the scientific community (Baulcombe, 1996; Cogoni et al., 1994; Fire et al., 1998; Kennerdell and Carthew, 1998; Ngô et al., 1998). RNAi is triggered by small-interfering RNAs (siRNAs) that are processed from long double-stranded or hairpin precursors and become part of the RNA-induced silencing complex (Lipardi et al., 2001; Sijen et al., 2001). siRNAs are expressed from DNA templates silence gene expression as effectively as exogenously introduced synthetic siRNAs (Brummelkamp et al., 2002; Paddison et al., 2002; Paul et al., 2002; Zeng et al., 2002). The use of RNAi has been extended to differentiated cultured mammalian cells (Elbashir et al., 2001; Kretschmer-Kazemi and Sczakiel, 2003), and successfully inhibits the replication of pathogenic viruses in culture, including the human immunodeficiency virus (HIV) (Bitko and Barik, 2001; Coburn and Cullen, 2002; Gitlin et al., 2002; Jacque et al., 2002). A series of different RNA or multiple shRNA based-inhibitors were developed for use in a gene therapy-based treatment of HIV-1 infection (Anderson et al., 2007; Barnor et al., 2005; Ter Brake et al., 2006; Li et al., 2006), but the emergence of siRNA-escape variants following siRNA administration in long-term cultures has been reported (Boden et al., 2003; Das et al., 2004; Westerhout et al., 2005).

In the present study, we designed an anti-HIV short-hairpin RNA (shRNA) that encodes a cleavable HIV-1 virion infectivity factor (vif) shRNA-decoy *trans*-activation response region (mini-TAR RNA) (Banerjea et al., 2004; Hamma and Miller, 1999; Huq et al., 1999; Li et al., 2005; Selby et al., 1989). The chimeric RNA expressed a dual RNA moiety and greatly enhanced the inhibition of HIV-1 replication under the production of resistant virus. The decoy TAR RNA domain engaged the HIV-1 tat protein in a competitive interaction, thereby attenuating the HIV-1 transcriptional *trans*-activation process (Fulcher and Jans, 2003; Michienzi et al., 2002). The expressed

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HIV-1 vif siRNA domain-mediated post-transcriptional degradation of HIV-1 cognate genes to achieve a dual inhibitory effect on HIV replication.

2. Materials and methods

2.1. Construction of U6 expression plasmids and lentiviral-based vectors

Expression plasmids were constructed using standard techniques. Hairpin shRNA sequences chemically synthesized as two complementary DNA oligonucleotides (see Supplementary Table 1) 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 KpnI and BamHI cloning sites upstream of the U6 promoter (Lee et al., 2002) of pSV2neo (TAKARA, Otsu, Japan). The U6 vif-shRNA TAR vector encodes both HIV-1 vif-shRNA and decoy TAR RNA; the U6 vif-shRNA vector encodes HIV-1 vif; the U6 vif-shRNA Ran vector encodes random vif; the U6 TAR vector encodes HIV-1 TAR; the U6 MTAR vector encodes a mutated TAR loop; the U6 vif-shRNA Ran-MTAR vector encodes random vif and mutated TAR.

To construct the lentiviral vectors, the EcoRI fragment of the U6 vectors listed above containing the U6 promoter and the siRNA duplex was cloned into the EcoRI site of the lentiviral transfer vector (pCS-CDF-CG-PRE) (Miyoshi et al., 1998), generating the CS-vif-shRNA TAR and control transfer vectors.

2.2. Cell culture

Peripheral blood mononuclear cells (PBMCs): human pooled PBMCs were isolated from four healthy HIV-seronegative donors by Ficoll-Paque Plus separation (Amersham Biosciences, Buckinghamshire, UK). The pooled PBMCs were cultured for 24 h and half the culture volume was replaced with the same volume of PBMCs

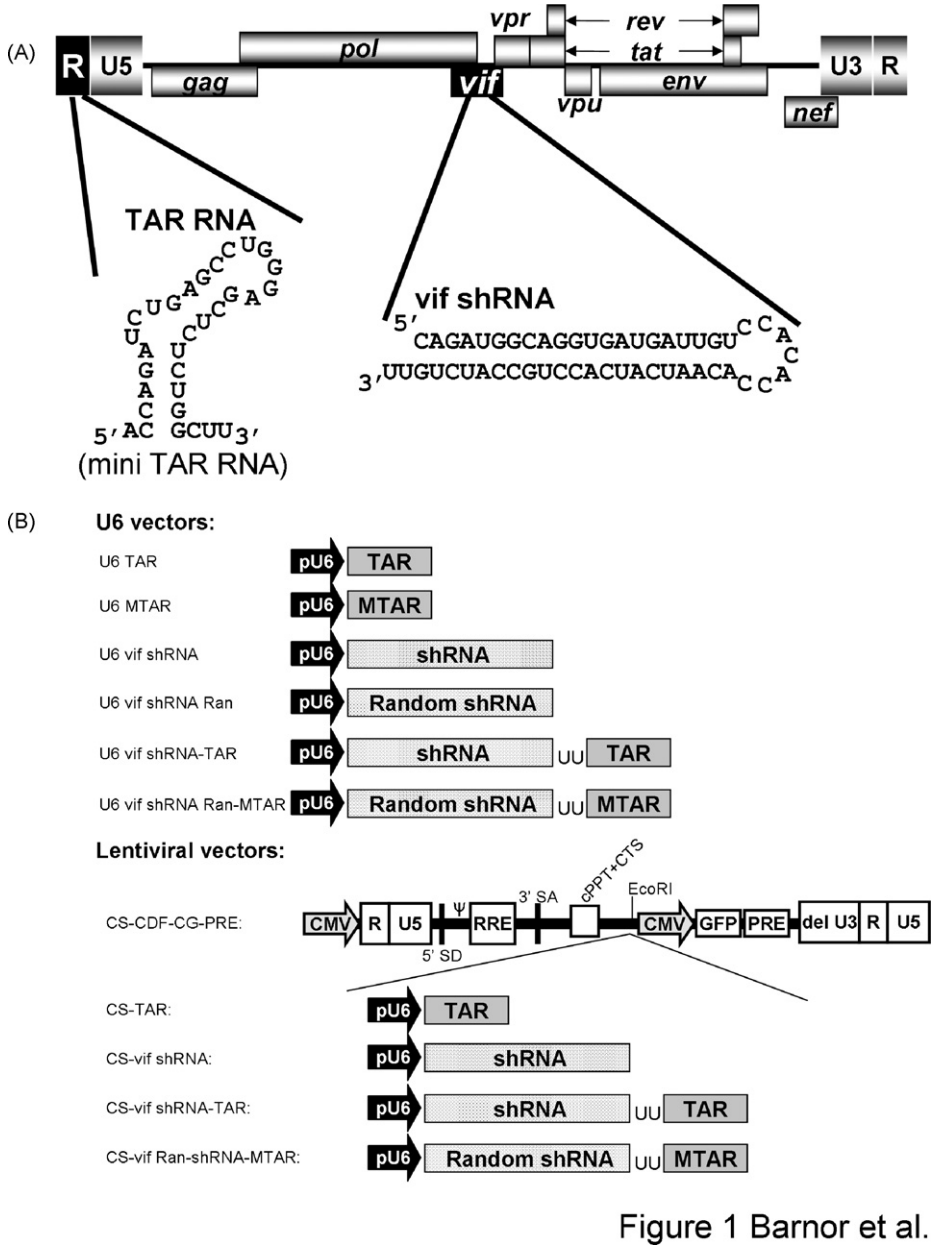

Figure 1 Barnor et al.

Fig. 1. Construction of second-generation shRNA expression vectors. (A) Schematic representation of HIV-1 genome showing decoy TAR RNA and vif-shRNA target sequences. (B) Schematic representation of human U6 promoter-driven U6-plasmid and CS-lentiviral vectors.

from a single donor (Vella et al., 1999). This process was repeated every 2 days for approximately 1 week before cells were used in the experiments. PBMCs, HeLa CD4$^+$, 293T, Jurkat, H9, and MT-4 cells were grown in either RPMI 1640 (Sigma, St. Louis, MO) 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.

2.3. In vitro and intracellular Dicer cleavage assay

Purified vif-shRNA TAR dsRNA (60 μg) transcribed with T7-RNA polymerase using a BLOCK-iT RNAi TOPO Transcription Kit (Invitrogen, Carlsbad, CA) was cleaved in a 300-μl reaction volume according to the manufacturer's protocol (BLOCK-iT Dicer RNAi Kit; Invitrogen). Briefly, the dicing reaction was incubated at 37 °C for 20 h, and the transcript was added to non-transfected control cell lysate for normalization. The products were resolved on a 20% (w/v) polyacrylamide gel (Invitrogen). HeLa CD4$^+$ cells were transfected with 3 μg each vector DNA using Lipofectamine 2000 reagent (Invitrogen), and total RNA was extracted with Trizol according to the manufacturer's instructions (Invitrogen). Both 72 h post-transfection and T7-transcribed RNA were subjected to Northern blot analysis.

2.4. Northern blot analysis

Total RNA was extracted from 5×10^5 HeLa CD4$^+$ cells after transient transfection using Trizol reagent according to the manufacturer's instructions (Invitrogen), and 30 μg samples were loaded onto a 20% (w/v) polyacrylamide/8 M urea gel. After transfer to a Hybond-N^{TM} nylon membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), synthetic oligonucleotides complementary to the antisense strand of the vif-shRNA-decoy TAR RNA were used as probes. Hybridization was performed at 37 °C, followed by washing with 2× SSPE at 39 °C and 1× SSPE at 41 °C, prior to autoradiographic exposure.

2.5. Dose-dependent inhibition of HIV-1 replication

The indicated U6 vectors (0.1, 1, and 3 μg; as in Fig. 1B) were co-transfected with 0.2 μg HIV-1pNL4-3-EGFP into 3×10^5 HeLa CD4$^+$ cells. The HIV-1pNL4-3-EGFP infectious molecular clone encoding EGFP (Miura et al., 2001) was based on the previously described HIV-1pNL4-3 (Adachi et al., 1986). Cell-free culture supernatants were harvested and extracellular HIV-1 gag p24 antigen production levels were measured as an index of viral replication.

2.6. Down-regulation of target mRNA and inhibition of HIV-1 replication

Total RNA from HeLa CD4$^+$ cells co-transfected with 2 μg vector DNA and 0.2 μg HIV-1pNL4-3-EGFP was extracted with Trizol reagent after 72 h of culture. The RNA content was examined using primer sets (forward: 5′-AAG TAG TGT GTG CCC GTC TGT TG-3′ and reverse: 5′-CTA GGA TCT ACT GGC TCC ATT TCT TGC-3′) that allowed for the detection of HIV-1 vif viral RNA, and for TAR RNA (forward: 5′-GCA ATG ATT GTC GTA ATT GC-3′ and reverse: 5′-CTT GCT CAG TAA GAA TTT TCG TC-3′). HIV-1 gag p24 antigen production levels were then measured in the cell-free harvested culture supernatants using a fully automated chemiluminescent enzyme immunoassay system (Fujirebio, Tokyo, Japan) (Sakai et al., 1999).

2.7. 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 (Stegmeier et al., 2005). Supernatants were harvested 72 h post-transfection, filtered through a 0.45 μm filter disc, and concentrated 100-fold by centrifugation at $6000 \times g$ 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 titer, 5×10^5 293T cells were transduced with the prepared viral stock, and the number of EGFP-positive cells was counted after 72 h culture using flow cytometric analysis (Ducrest et al., 2002).

2.8. Transduction of PBMCs and H9 cells

Human PBMCs (1×10^6), H9 cells (2×10^5), and Jurkat cells (2×10^5) were seeded in 12-well plates in 1 ml culture medium. Cells were transduced with the CS-vif-shRNA TAR 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 8–16 h, the medium was removed before the HIV-1 challenge was initiated.

2.9. IFN-β ELISA

Human IFN-β was detected in culture supernatants using a Human Interferon Beta (Hu-IFN-β) ELISA Kit (PBL Biomedical Laboratories, Piscataway, NJ) following the manufacturer's instructions. For control IFN production, 1 μg poly I:C was transfected into PBMCs with Lipofectamine 2000 according to the manufacturer's instructions. Transduction of lentiviral vectors is described above. Culture supernatants were assayed 2 days after transfection of poly I:C or transduction of lentiviral vectors.

2.10. Generation of viruses

To generate HIV-1 viruses, the HIV-1 infectious molecular cloned plasmid vector (HIV-1pNL4-3) was transfected (3 μg DNA) into 24-h seeded HeLa CD4$^+$ cells (5×10^5) using Lipofectamine 2000 according to the manufacturer's instructions. The culture was incubated at 37 °C for 72 h, then harvested and the cells pelleted by centrifugation to produce the cell-free supernatant yielding the HIV-1_{NL4-3} virus, which was aliquoted and stored at −80 °C. HIV-$1_{NL4-3-vif-mut}$ virus was generated from the experiment shown in Fig. 4B. Briefly, PBMCs transduced in the presence of 4 μg/ml polybrene with lentivirus-mediated vif-shRNA at an MOI of 20 and challenged with HIV-1_{NL4-3} virus at an MOI of 0.01 were cultured for 9 weeks at 37 °C. At week 6, harvested supernatant showing a vif mutation virus HIV-$1_{NL4-3-vif-mut}$ (siRNA vif target 5049-CAGATGGCAGGTGATGATTGT-5069; vif-shRNA/3 weeks post-infection, **AG**G-TGGC**GA**—ATGATT**A**T: 5 nt substitutions and 4 deletions), was titered and stocked at −80 °C and later used as the HIV-$1_{NL4-3-vif-mut}$ virus.

2.11. HIV-1 challenge and long-term culture assay

After transduction, PBMCs, H9 cells, and Jurkat cells expressing the transgenes were challenged with HIV-1_{NL4-3} at an MOI of 0.01. Following infection, the cells were washed three times with phosphate-buffered saline (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. One-half of the culture volume was harvested and replaced with an equal volume of culture medium at regular intervals. The harvested culture was centrifuged and the cell-free

medium 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.

2.12. Flow cytometry analysis of long-term EGFP expression

One-half volume of culture was harvested 2, 4, 6, and 8 weeks post-challenge, pelleted, washed twice in PBS, and resuspended in 1% formaldehyde. FACS analysis was performed using the FACSCalibur and *CELLQUEST* software (BD Sciences, San Jose, CA).

2.13. Genotypic sequence analysis of the vif siRNA target region of HIV-1_{NL4-3} and rechallenge of wild-type vif-shRNA TAR expressing cells

Viral RNA from HIV-1_{NL4-3}-challenged CS-vif-shRNA TAR or CS-vif-shRNA-transduced cultures was analyzed for siRNA-mediated mutations in the vif-shRNA target region at weeks 2, 4, 6, and 8, as described previously (Sijen et al., 2001). Viral RNA was isolated from the cell-free culture supernatant using a QIAamp viral RNA kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Viral RNA (5 μl) was used in a reverse transcription-polymerase chain reaction (PCR) reaction containing Powerscript reverse transcriptase (Clontech, Mountain View, CA), 1 μM each of the deoxynucleotide triphosphates, 1× first-strand buffer (Clontech), 200 ng random hexamer (Promega, Madison, WI), 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 vif F: (5′-ATG GAA AAC AGA TGG CAG GTG AT-3′), and antisense primer vif R: (5′-CTA GTG TCC ATT CAT TGT ATG GCT-3′), 1 mM each of the deoxynucleotide triphosphates, and 2.5 U Taq polymerase (Qiagen). PCR was performed in a gradient PCR thermal cycler (Astec, Fukuoka, Japan) using the following thermal program: 1 cycle (95 °C for 1 min), 35 cycles (95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30), and 1 cycle (72 °C for 5 min). The PCR product was fractionated and analyzed 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.

PBMCs stably expressing vif-shRNA-decoy TAR RNA, vif-shRNA, decoy TAR RNA, and the control (PBMCs, U6-ter) were challenged with either wild-type virus HIV-1_{NL4-3} or mutant virus HIV-$1_{NL4-3-vif-mut}$. Human PBMCs (1×10^6) were infected with 20 ng p24 of 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 a 5-week period by HIV-1 gag p24 ELISA.

2.14. Statistical analysis

Statistical analysis was performed using a one-tailed Student's *t*-test. A *p*-value of less than 0.05 was considered significant. Data were based on means ± standard error (SE) of three separate experiments performed in duplicate.

3. Results

3.1. Construction of U6 expression plasmids and lentiviral-based vectors, and Dicer cleavage assay

We constructed vectors that specifically target the vif and TAR sequences of HIV-1 (Fig. 1A): the pSV2neo-U6-plasmids (U6) and pCS-CDF-CG-PRE-based lentiviral vectors (CS) (Fig. 1B), and

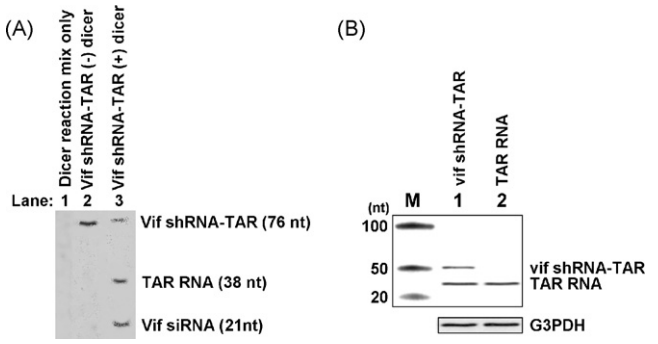

Fig. 2. Predicted secondary RNA structure, expression, and cleavage. (A) *In vitro* cleavage of RNA substrate. Northern blot analysis of T7-RNA polymerase-transcribed vif-shRNA-decoy TAR RNA (60 μg) treated with human recombinant Dicer. Lane 1: negative control without Dicer or sample; lane 2: vif-shRNA TAR RNA without Dicer detected with ^{32}P tagged vif-shRNA and TAR probe; lane 3: vif-shRNA TAR RNA with Dicer detected with ^{32}P tagged vif-shRNA and TAR probe. (B) Intracellular expression of vector RNA. Intracellular cleavage of chimeric RNA (vif-shRNA TAR) shown by Northern blot analysis of total RNA extracted 72 h post-transfection from HeLa $CD4^+$ cells. M: RNA size marker; lane 1: vif-shRNA TAR RNA detected with ^{32}P tagged TAR probe; lane 2: expressed decoy TAR RNA as an internal control marker detected with ^{32}P tagged TAR probe. G3PDH was used as an internal control and loading standard in both experiments.

analyzed the predicted RNA secondary structure of the vif-shRNA-decoy TAR RNA molecule using GENETYX software. Intracellular expression of U6 vif-shRNA TAR and control vector RNAs (U6 vif-shRNA, U6 TAR, U6 vif-shRNA Ran, U6 MTAR, and U6 vif-shRNA Ran-MTAR) was confirmed by Northern blot analysis after lipid transfection of HeLa $CD4^+$ cells (data not shown).

The RNAi mechanism is closely linked with the activity of the endogenous RNase III-like enzyme, Dicer (Kawasaki and Taira, 2003). To evaluate the cleavage efficacy of the vif-shRNA TAR RNA molecule, we incubated the transcribed substrate with human recombinant Dicer and subjected it to Northern blot analysis. This revealed high cleavage efficacy compared with the control non-Dicer cleaved vif-shRNA TAR RNA product (Fig. 2A). Furthermore, we investigated the *in vivo* processing of vif-shRNA-decoy TAR RNA into siRNAs and decoy TAR RNAs by endogenous Dicer in transfected HeLa $CD4^+$ cells. Northern blot analysis revealed that the vif-shRNA TAR RNA molecule was cleaved (Fig. 2B). However, the function as a decoy TAR RNA in chimeric RNAs was demonstrated by vif-shRNA-decoy TAR RNA present in the nucleus.

3.2. Inhibition of HIV-1 gene expression by shRNA and decoy RNA

To explore the dose-dependent inhibitory efficacy of the vif-shRNA TAR RNA molecule on HIV-1 replication, HIV-1 gag p24 antigen levels were measured in HeLa $CD4^+$ cells that were co-transfected with U6-ter, U6 vif-shRNA TAR RNA, U6 vif-shRNA, U6 vif-shRNA Ran, U6 TAR or U6 vif-shRNA Ran MTAR. U6 vif-shRNA TAR transfection inhibited HIV-1 replication in a dose-dependent manner with a greater than 90% maximum inhibitory efficacy for 72 h (Fig. 3A). Both U6 vif-shRNA TAR RNA and U6 vif-shRNA generated almost equally high inhibition. Furthermore, the level of enhanced green fluorescent protein (EGFP) expression, used as an index of replication, was notably reduced in transfected HeLa $CD4^+$ cells (Fig. 3B).

3.3. Long-term inhibition of HIV-1 replication by lentiviral vector-mediated shRNA-decoy RNA

Lentiviral (CS-vector) versions of the plasmid U6 vectors (Fig. 1B) were generated to enhance the delivery and durability of the RNA chimera by utilizing their ability to transduce non-dividing T-cells

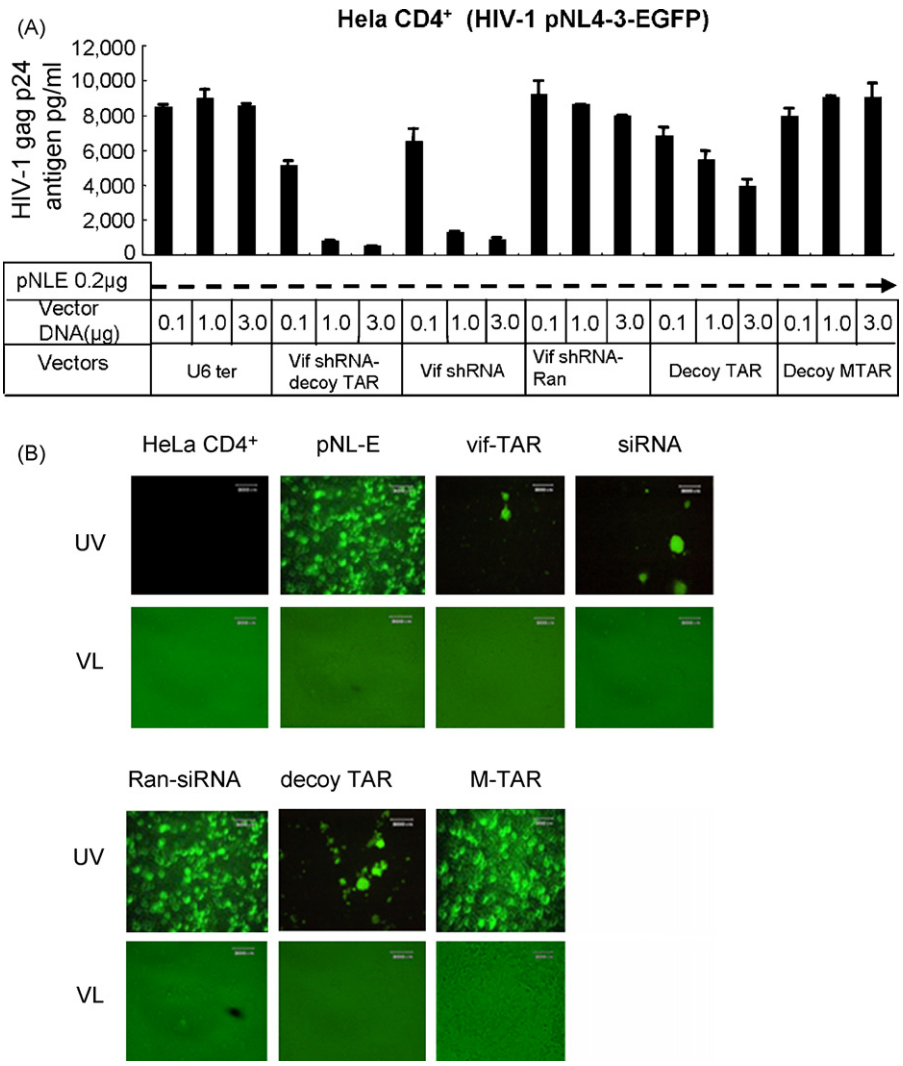

Fig. 3. Anti-viral efficacy of vector constructs. (A) HeLa CD4+ cells co-transfected with different concentrations of indicated U6 vectors and 0.2 μg HIV-1 pNLE4-3 were tested for HIV-1 gag p24 antigen expression using an automated ELISA system. Data represent mean values ± standard deviation (SD) of three independent experiments. (B) Down-regulation of reporter gene expression in HeLa CD4+ cells co-transfected with indicated U6 vectors and HIV-1 pNLE4-3 was examined under ultraviolet light (UV) and visible light (VL) to evaluate EGFP expression as an index of down-regulation of HIV-1 replication.

(Huq et al., 1999). Peripheral blood mononuclear cells (PBMCs), H9 cells, and Jurkat cells were stably transduced with these vectors. To determine whether vif-shRNA TAR was immunostimulatory, we transduced PBMCs with a lentiviral vector (CS-vif-shRNA TAR). An enzyme-linked immunosorbent assay (ELISA) for interferon (IFN)-β protein was performed on cell supernatants. IFN-β protein was not detected in supernatants from control cells or parental CS-CDF-CG-PRE (CS-blank) and CS-vif-shRNA TAR (CS-vif-TAR) transduced cells (Fig. 4A). These transduced cells were challenged with HIV-1_{NL4-3}, and HIV-1 gag p24 antigen levels were measured as an index of viral replication or inhibition by the expressed transgenes. The siRNA related-escape mutant phenomenon was observed at 3 weeks in the transduced PBMCs, as indicated by the virus breakthrough effect (Fig. 4B), compared with 4 weeks in H9 cells (Fig. 4C). In both PBMCs and H9 cells, CS-vif-shRNA TAR expressed RNA-mediated stable inhibition of HIV-1 replication was observed with the RNAi-resistant virus at 9 weeks (Fig. 4B and C). Similar inhibition levels were observed in both transduced cell types expressing the TAR RNA. A steady increase in viral expression was maintained until week 4, then fluctuations in inhibition efficacy were observed from week 4 to 9 in the H9 cells (Fig. 4C), and from week 5 to 9 in the PBMCs (Fig. 4B). Furthermore, to determine whether HIV-1 was down-regulated by APOBEC3G, APOBEC3G-defective Jurkat cells transduced with CS-vif-shRNA TAR were tested for HIV-1 gag p24 antigen expression. Similar results were observed in the APOBEC3G-expressing H9 cells (Fig. 4C) and APOBEC3G-defective Jurkat cells (Fig. 4D). To quantitatively estimate the duration of gene expression, we performed a time-course experiment that revealed EGFP expression at weeks 2, 4, 6, and 8 in both transduced PBMCs and H9 cells. EGFP expression persisted up to week 8 (Fig. 4E).

3.4. RNAi-resistant HIV-1 variants

siRNAs targeted to HIV genes in long-term stably expressing cultures give rise to escape mutants (Boden et al., 2003; Das et al., 2004; Westerhout et al., 2005). We therefore investigated the sudden upsurge in viral replication in cultures expressing vif-shRNA. Sequence analysis revealed that both cultures expressing vif-shRNA alone and vif-shRNA TAR RNA showed resistance against vif-shRNA (Fig. 5A). The vif-shRNA TAR, however, effectively inhibited 98% of HIV-1 replication at 9 weeks. Most surprisingly, there was an emergence of RNAi-resistant viruses that contained nucleotide substitutions or deletions in or near the shRNA-vif target sequence. Partial substitutes or deletions were observed in six cultures at 2

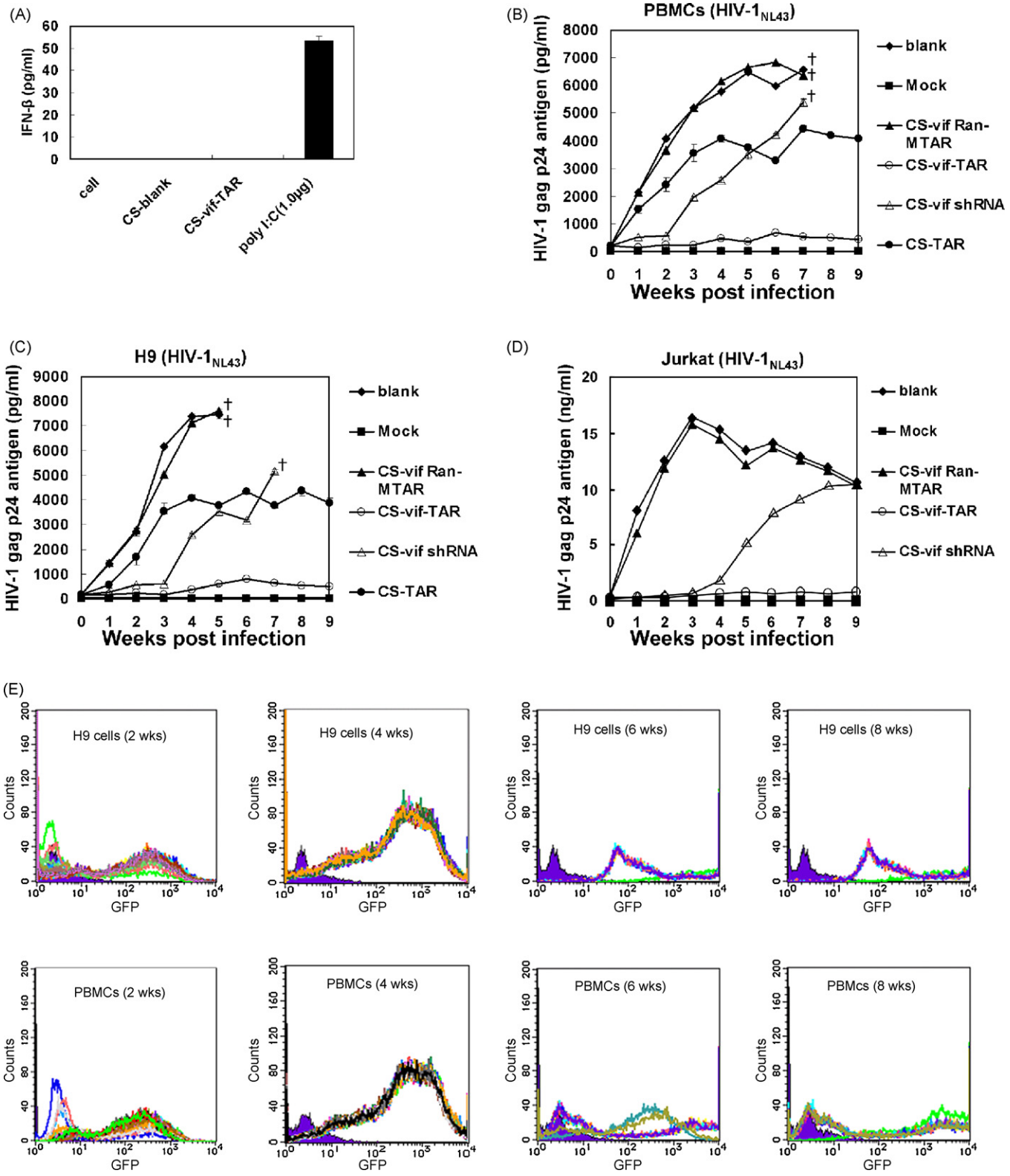

Fig. 4. Side effect and long-term activity of lentivirus-mediated vif-shRNA TAR RNA. (A) IFN-β ELISA was performed on supernatants. Data are from triplicate experiments. (B) Long-term inhibition of HIV-1 replication in PBMCs. HIV-1 gag p24 antigen expression was measured during the 9-week culture of PBMCs transduced with indicated CS-lentiviruses (MOI 20) and challenged with HIV-1_{NL4-3} (MOI 0.01). Data are from duplicate experiments. HIV-1 gag p24 antigen expression was measured during the 9-week culture of H9 (C) and Jurkat (D) cells under same experimental conditions as described for (B). (E) Long-term expression of transgenic EGFP expression in PBMCs and in H9 cells expressing vector transgenes was examined by FACS analysis using *CELLQUEST* software. Data are from duplicate experiments.

(A)

Vector	Week	Clone	siRNA-vif target	Nucleotide substitution / deletion
			5049 5069	
HIV-1 $_{NL4-3}$			CAGATGGCAGGTGATGATTGT	
shRNA	2	#1	CAGAGGACAGATGGAACAAGC	10 nt substitutions
	2	#2	CAGAGGACAGATGGAACAAGC	10 nt substitutions
	2	#3	CCTTGGGCAGGTGATGATTGT	4 nt substitutions
vif shRNA-decoy TAR	2	#1	CAGATGGCAGGTGATGATTGT	
	4	#1	CAGAGGACAGATTAACAAGCC	10 nt substitutions
	4	#2	TATCAAGCAGGACATAAGCAA	11 nt substitutions
	4	#3	CAGAGGACAGATGGAACA-GC	9 nt substitutions and 1 deletion

(B)

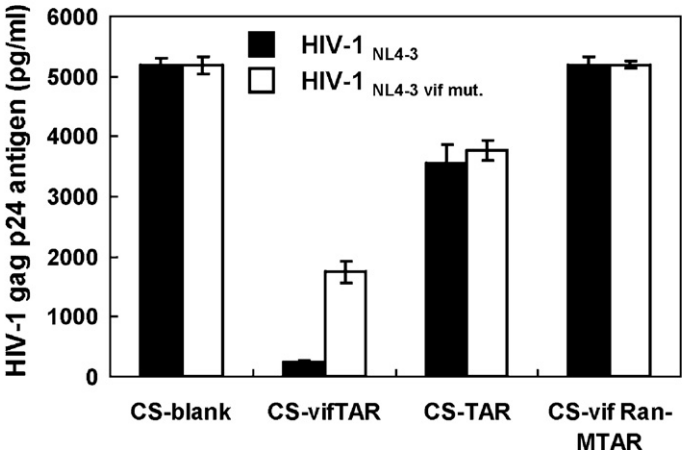

Fig. 5. HIV-1 escape variants that resist vif siRNA inhibition. (A) Genotype sequence analysis revealed siRNA-mediated mutations in vif-shRNA target site (nucleotides 5049–5069) of HIV-1_{NL4-3} in RNA extracted from vif-shRNA and vif-shRNA TAR RNA-expressing culture supernatants. Day at which escape variants were sequenced is indicated. Deletions are shown as dashes, substitutions are underlined and in bold. (B) PBMCs stably expressing vif-shRNA-decoy TAR RNA, vif Ran-shRNA-decoy MTAR RNA, and decoy TAR RNA cells infected with mutant virus HIV-$1_{NL4-3-vif-mut}$ and wild-type virus HIV-1_{NL4-3} over a 3-week period. Viral challenge of vif-shRNA-TAR RNA-expressing cells with wild-type virus HIV-1_{NL4-3} and mutant virus HIV-$1_{NL4-3-vif-mut}$ resulted in 98% and 68% inhibition of HIV-1 replication. Decoy TAR RNA alone showed the low inhibition of both HIV-$1_{NL4-3\ 3}$ and HIV-$1_{NL4-3-vif-mut}$ infections. Viral challenge of the control, vif Ran-shRNA-decoy MTAR RNA-expressing cells with HIV-$1_{NL4-3-vif-mut}$ and HIV-1_{NL4-3} did not result in inhibition of HIV-1 replication. Data are from duplicate experiments.

weeks (vif-shRNA) and 4 weeks (vif-TAR shRNA). No mutations in the *tat* sequence were observed (data not shown).

To determine whether the viral escape mutant was indeed resistant to vif-shRNA, we took PBMCs stably expressing vif-shRNA-decoy TAR RNA, vif Ran-shRNA-decoy MTAR RNA, and decoy TAR RNA and infected them with the evolved HIV-$1_{NL4-3-vif-mut}$ and wild-type HIV-1_{NL4-3}. HIV-1_{NL4-3} caused 98% inhibition of HIV-1 replication in the vif-shRNA-decoy TAR RNA-expressing PBMCs at 3 weeks (Fig. 5B), whereas 68% inhibition was observed from mutant virus HIV-$1_{NL4-3-vif-mut}$ at the same time period. Further, decoy TAR RNA alone demonstrated 37% inhibition of HIV-1 replication, and its inhibitory effect was essentially due to the decoy TAR RNA. By contrast, viral challenge of the control, vif-Ran-shRNA-MTAR expressing PBMCs with HIV-$1_{NL4-3-vif-mut}$ and HIV-1_{NL4-3} did not result in the suppression of viral replication. These results demonstrate that the efficiency of siRNA-binding to target RNA can be diminished by nucleotide substitutions or deletions in the target sequence. These mutations presumably induce an alternative secondary structure in the RNA genome that reduces the efficiency of RNAi (Westerhout et al., 2005).

4. Discussion

RNAi is a potent inhibition technique that shows great promise for the treatment of HIV/AIDS. The sensitivity of the target region to RNAi, however, can lead to the emergence of RNAi-resistant HIV mutants (Boden et al., 2003; Das et al., 2004; Westerhout et al., 2005). Sequencing of emerging RNA-resistant viruses previously revealed alterations in the *nef* sequence; in some cases the siRNA recognition site contained several nucleotide substitutions that disrupted base-pairing and in other cases the target was deleted (Das et al., 2004). Analogous to the current clinical use of combinations of anti-viral drugs that target reverse transcriptase and protease enzymes, we propose that a combination of different therapeutic RNAs inhibiting multiple steps in the viral life cycle might be the most efficacious way to treat this infection in a gene therapy setting.

We investigated the intracellular processing of vif-shRNA-decoy TAR RNA into siRNAs and decoy TAR RNAs by endogenous Dicer in transfected HeLa CD4$^+$ cells. The vif-shRNA TAR RNA molecule was cleaved at 72 h (Fig. 2B). The first step in the HIV-1 inhibitory effect of these RNA molecules may arise from the decoy TAR RNA moiety of intranuclear RNA. Subsequently, the cytoplasmic vif siRNA cleavage product is expected to exert anti-HIV-1 activity by enhancing the inhibition of HIV-1 replication in infected cells. In this study, we demonstrated that each of the therapeutic RNA agents, vif-shRNA and decoy TAR RNA, and the vif-shRNA-decoy TAR RNA, had anti-HIV-1 activity (Fig. 3). On the other hand, decoy TAR RNA demonstrated a slightly reduced inhibitory effect on HIV-1 replication compared with vif-shRNA transfected cells. The vif-shRNA-decoy TAR RNA construct was effective for co-suppression of HIV-1 replication. As transfected shRNA plasmid vectors are likely to have only a transient effect, it will be necessary to use methodologies that induce constitutive expression in target cells to produce a sustained effect. To this end, the use of retroviral or lentiviral vectors to deliver shRNAs into cells would be ideal. Such a goal was accomplished in recent studies of lentiviral transduction of anti-siRNA into hematopoietic stem cells from which HIV-1-resistant T-cells and macrophages were derived (Hamma and Miller, 1999; Li et al., 2005).

We demonstrated that these inhibitors act as Pol III expression units within the lentiviral vector backbone. In both PBMCs and H9 cells, stably expressed CS-vif-shRNA TAR RNA inhibited HIV-1 replication with an RNAi-resistant virus at 9 weeks (Fig. 4B and C). Hence, siRNAs targeted to HIV genes in long-term stably expressing cells give rise to escape mutants, but RNAi-resistant HIV-1 was suppressed by decoy TAR RNA. There was no difference in 9-week p24 antigen suppression levels between transduced H9 cells (Fig. 4C). Moreover, a similar inhibitory effect of CS-vif-shRNA TAR RNA was observed in APOBEC3G-defective Jurkat cells (Fig. 4D). These results suggest that APOBEC3G had no effect in this vif-shRNA TAR RNA study. Further, IFN-α protein was not detected in the supernatant from control cells or CS-vif-shRNA TAR-transduced cells (Fig. 4A). This supports previous findings by Robbins et al. who demonstrated that IFN-β was not induced in lentiviral vector-transduced $CD34^+$ progenitor cells (Robbins et al., 2006).

Despite the inhibitory action of the single vif-shRNA, the siRNA related-escape mutant phenomenon was observed 3 weeks in transduced PBMCs as indicated by the virus breakthrough effect (Fig. 4B), compared with 4 weeks in H9 cells (Fig. 4C). Most surprisingly, RNAi-resistant viruses emerged that contained nucleotide substitutions or deletions in or near the shRNA-vif target sequence (Fig. 5A). The efficiency of RNAi-mediated inhibition depends on the efficiency of siRNA-binding to the target RNA. This interaction can be diminished by nucleotide substitutions or deletions in the target sequence that cause a mismatch with the siRNA or by mutations that induce a secondary RNA structure in which the target sequence is occluded (Westerhout et al., 2005). Furthermore, viral challenge of vif-shRNA TAR-expressing cells with mutant virus $HIV\text{-}1_{NL4\text{-}3\text{-}vif\text{-}mut}$ resulted in 68% suppression of HIV-1 replication (Fig. 5B), but cells with only decoy TAR RNA induced a low level of HIV-1 inhibition. The addition of the stem and hairpin loop region to the decoy TAR RNA sufficiently stabilized the structure of the decoy RNA to increase its HIV-1 inhibiting activity.

Decoy TAR RNA function in chimeric RNA is demonstrated by the interaction of vif-shRNA-decoy TAR RNA with HIV-1 tat protein present in the nucleus. Non-interacting vif-shRNA-decoy TAR RNA was exported to the cytoplasm, so the decoy RNA appears to contribute much less to the overall inhibition than the shRNA. Even so, decoy RNA activity was immediately restored by incorporating the TAR sequences into the shRNA-decoy RNA. We did not, however, observe any mutations in the *tat* sequence (data not shown). There was no difference in p24 antigen suppression levels at 9 weeks in either of the transduced H9 cells.

In conclusion, this study provides strong evidence that the development of a combination gene therapy strategy could have therapeutic importance for the delivery of multiple genes and the enhancement of inhibitory efficacy. The advantage of the lentiviral vector is that the anti-viral RNAs, shRNA and RNA decoy inhibit HIV-1 via different mechanisms. Future studies and improvements are needed to enhance the potential of this strategy as a novel method for siRNA-based HIV-1 gene therapy in the treatment of HIV/AIDS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2009.04.008.

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