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Lentiviral Vector Design for Multiple shRNA Expression and Durable HIV-1 Inhibition

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Human immunodeficiency virus type 1 (HIV-1) replication in T cells can be inhibited by RNA interference (RNAi) through short hairpin RNA (shRNA) expression from a lentiviral vector. However, for the development of a durable RNAi-based gene therapy against HIV-1, multiple shRNAs need to be expressed simultaneously in order to avoid viral escape. In this study, we tested a multiple shRNA expression strategy for different shRNAs using repeated promoters in a lentiviral vector. Although highly effective in co-transfection experiments, a markedly reduced activity of each expressed shRNA was observed in transduced cells. We found that this reduced activity was due to recombination of the expression cassette repeat sequences during the transduction of the lentiviral vector, which resulted in deletions of one or multiple cassettes. To avoid recombination, we tested different promoters for multiple shRNA expression. We compared the activity of the human polymerase III promoters U6, H1, and 7SK and the polymerase II U1 promoter. Activities of these promoters were similar, irrespective of which shRNA was expressed. We showed that these four expression cassettes can be combined in a single lentiviral vector without causing recombination. Moreover, whereas HIV-1 could escape from a single shRNA, we now show that HIV-1 escape can be prevented when four shRNAs are simultaneously expressed in a cell.

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INTRODUCTION

RNA interference (RNAi) through transient transfection of small interfering RNA (siRNA) is highly effective against a wide range of viruses, for instance human immunodeficiency virus type 1 (HIV-1), hepatitis B virus, hepatitis C virus, influenza virus A, and several coronaviruses.^{1,2} A phase I clinical trial has already been performed for respiratory syncytial virus infection with nasally administered siRNA.³ However, a constant supply of siRNA is required for the development of an RNAi-based therapy for chronic viral infections like HIV-1. An elegant way to achieve this objective is to use a gene therapy approach in which the antiviral siRNA is stably expressed in the cell as a short hairpin RNA

(shRNA).⁴ Stable expression of anti-HIV shRNAs in T cells results in potent inhibition of HIV-1 replication.^{5,6} However, the application of a single shRNA inhibitor is not sufficient to maintain inhibition. After extended culturing, resistant virus can emerge containing point mutations or deletions within the siRNA target site.⁷⁻¹¹ Thus, in order to achieve durable inhibition, multiple antiviral siRNAs should be expressed simultaneously,^{9,12,13} a strategy similar to current drug combination therapy of HIV-1-infected individuals.^{14,15} Strategies to express multiple siRNAs¹⁶ include extended shRNAs,¹⁷ long double-stranded RNA expression,¹⁸ and multiple shRNA expression,^{9,19} of which the latter is currently most promising.

The lentiviral vector system is highly effective for delivery of shRNA expression cassettes.²⁰⁻²⁴ An apparently straightforward method to express multiple shRNAs from a lentiviral vector is to incorporate multiple expression cassettes that use the same promoter.^{6,9,19,25,26} However, such an approach may be problematic due to intrinsic properties of the lentiviral vector. Retroviruses are naturally recombination prone, as the reverse transcriptase is a polymerase with poor processivity. During the transduction process, in which reverse transcriptase converts the RNA genome into DNA, reverse transcriptase can slip on repeats in the RNA template, resulting in a sequence duplication or deletion.²⁷⁻³⁰ This could seriously hamper the genetic stability of lentiviral vectors with multiple shRNA cassettes.

We constructed single, double, and triple anti-HIV shRNA lentiviral vectors that express the shRNAs from the same repeated promoters. Initial co-transfection experiments indicate that such a multiple shRNA expression strategy is effective because the individual shRNA activity was comparable for single and multiple shRNA constructs. However, a strikingly different pattern emerged in transduced cells, since the shRNA activity was diminished with an increasing number of shRNA cassettes. We analyzed cell clones transduced with the multiple shRNA lentiviral vectors and found that recombination occurs frequently. For instance, only 14% of the cell clones transduced with a triple shRNA lentivirus contained an intact provirus. Thus, the reduced shRNA activity was caused by a recombination-mediated deletion of shRNA cassettes. In order to prevent recombination, we designed a viral vector without repeat sequences. Four different shRNAs were expressed from four different promoters. We analyzed transduced

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cell clones and confirmed that deletion did not occur. Finally, we show for the first time that HIV-1 escape can be prevented when four different shRNAs are simultaneously expressed.

RESULTS

Reporter gene knock-down is reduced in a triple shRNA cell line

We screened 86 shRNAs targeting highly conserved HIV-1 sequences.⁹ We identified multiple potent inhibitors of HIV-1 that were validated for sequence specificity with Luciferase reporters (Figure 1a). For the development of a multiple shRNA approach, we constructed plasmids encoding two or three different shRNAs, each expressed from their own H1 promoter (Figure 1b). The shRNA2 construct encodes the Pol-47 and Gag-5 inhibitors, shRNA3 encodes Pol-1 in addition. We used appropriate Luciferase reporters to measure the activity of individual shRNAs. In initial co-transfection experiments, in which reporters for each

shRNA target were co-transfected with the shRNA1, shRNA2, and shRNA3 plasmids, we measured specific reporter gene knock-down irrespective of the type of vector used. For instance, inhibition of Pol-47 was scored on the Luc Pol-47 reporter, showing equal activity of shRNA1, shRNA2, and shRNA3 (Figure 1c). Several control vectors, including the empty lentiviral vector JS1 and several unrelated shRNAs (Gag-5 and Pol-1) have no impact on the Luc Pol-47 reporter. Similar results were obtained for the other shRNAs in the multi-shRNA vectors on the matching reporters (data not shown).

Next, we transduced 293T cells with these lentiviral vectors at a low multiplicity of infection (MOI) of 0.15 to obtain a single lentiviral vector copy per cell. Transduced green fluorescent protein (GFP) positive cells were selected with live fluorescence-activated cell sorting (FACS). We transfected these cells with the Luc Pol-47 reporter and observed good inhibition for shRNA1, but a profound decrease in the magnitude of inhibition for shRNA2 and shRNA3 (Figure 1d). A similar drop in activity was scored for the other shRNAs encoded by the shRNA2 and shRNA3 constructs on their respective targets (data not shown). Thus, a striking difference was observed in the transiently transfected cells versus the stably transduced cell lines. Whereas full activity of the shRNAs was observed in the former assay, the inhibitory potential of shRNA2 and shRNA3 is significantly reduced in the transduced cells. One could argue that this is due to saturation of the RNAi pathway, but this is highly unlikely because the transduced cells will only have a single lentiviral vector copy. Another possibility is that not all shRNA cassettes were effectively transduced, *e.g.*, through recombination-mediated deletion due to the repeat sequences in the expression cassette (Figure 1b).²⁷

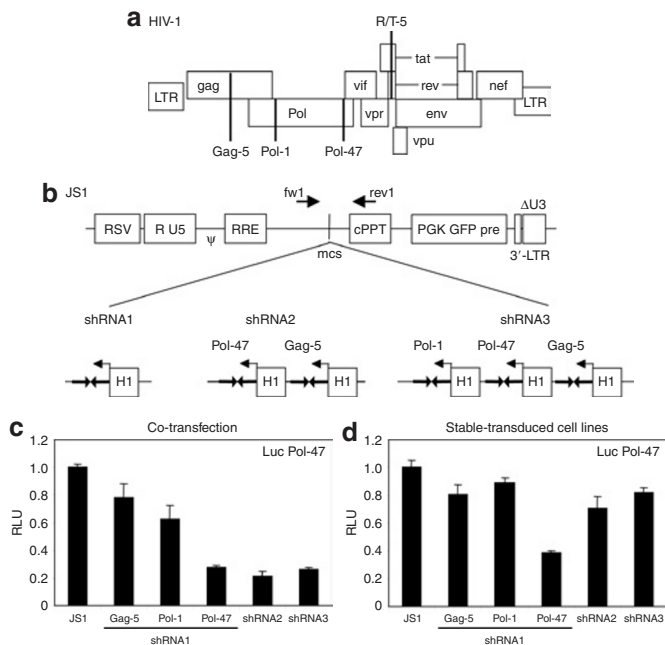


Figure 1 Reduced reporter knock-down in multi-short hairpin RNA (shRNA)-transduced cells. **(a)** Conserved shRNA target sequences in the human immunodeficiency virus type 1 (HIV-1) genome used in this study. **(b)** Lentiviral vector constructs used in this experiment. Single (shRNA1: Gag-5, Pol-1, or Pol-47), double (shRNA2), and triple shRNA (shRNA3) expression cassettes were cloned in the multiple cloning site (mcs) of JS1, the lentiviral vector construct. The lentiviral vector is a third generation self-inactivating vector, which contains an enhanced green fluorescent protein (eGFP) expression cassette that allows live sorting. Primers used to amplify across the mcs are indicated (fw1 and rev1). **(c)** A Luciferase reporter for Pol-47 was co-transfected in 293T cells with the empty control vector (JS1) or vectors expressing a single shRNA against HIV-1 (Gag-5, Pol-1, Pol-47), and vectors with a double and triple shRNA cassette (shRNA2 or shRNA3) and relative Luciferase expression was measured. **(d)** 293T cells were transduced with the indicated lentiviral vectors at a multiplicity of infection of 0.15 and selected with live fluorescence-activated cell sorting for GFP. Cells were transfected with the Luciferase reporter for Pol-47 and relative luciferase expression was measured. Averages and SDs in **c** and **d** represent three independent transfections, the average activity of the JS1 control was set at 1.0. LTR, long-terminal repeat; RLU, relative light units; RRE, rev-responsive element; RSV, Rous sarcoma virus promoter.

Deletion of shRNA cassettes from the multi-shRNA vectors

The repeat sequences present in the triple cassette insert constitute the H1 promoter [230 nucleotides (nt)] and the sequence encoding the pol III terminator (28 nt) (Figure 2a). To test for the intactness of the integrated provirus, we selected cell clones of transduced 293T cells and amplified the shRNA insert in a polymerase chain reaction (PCR) with primers fw1 and rev1 (Figure 1b). We analyzed 22 shRNA3 clones. Control untransduced (293T) and shRNA1-transduced cell clones were included, as were the input plasmids for shRNA1, shRNA2, and shRNA3. Strikingly, the observed bands for the shRNA3 cell clones differed among the different clones with discrete sizes corresponding to either a single, double, or triple cassette (Figure 2b). Only the minority of cell clones seem to have an intact triple cassette (3 of 22 cultures, 14%), whereas we observed 12 apparent doubles and 7 singles.

We sequenced the PCR products to analyze the actual recombination event (summarized in Figure 2c). If we look at the single-cassette deletions, cassette B was most frequently deleted (9 of 12 clones). Cassette A and C were only deleted once and twice, respectively. For the double-cassette deletion mutants, both AB (3×) and BC (4×) deletions were observed. No proviruses were observed in which A and C are deleted, probably because it requires a double recombination event. Deletion of all three cassettes is not possible by repeat-induced recombination, and is

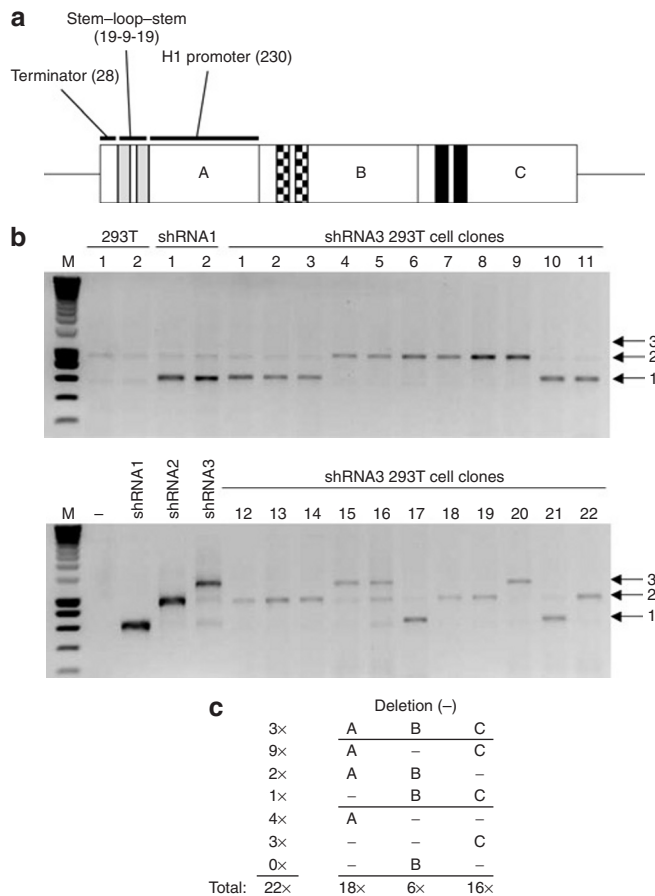


Figure 2 Triple short hairpin RNA (shRNA)-transduced cell lines contain cassette deletions. **(a)** Schematic representation of repeat sequences present in the shRNA3 lentiviral vector construct. The different shRNA expression cassettes for Pol-1, Pol-47, and Gag-5 are marked with A, B and C. The H1 promoter repeat sequence is 230 nucleotide (nt) in size, the sequence containing the pol III termination signal is 28 nt (terminator). The difference between the three cassettes is the shRNA encoding sequence, stem-loop-stem (gray, A; blocked, B; black, C). **(b)** 293T cells were transduced with shRNA3 at a multiplicity of infection of 0.15 and cell clones were selected with live fluorescence-activated cell sorting. A polymerase chain reaction (PCR) was performed across the cassette insertion. Control PCRs for 293T and shRNA1 clones were included, as well as an empty control (-) and input plasmids (shRNA1, 2, and 3). Differently sized PCR products were observed in the cell clones corresponding in size to either a single, double, or triple cassette insert. **(c)** Overview of sequence analysis of the shRNA3 293T cell clones. All possible deletions are shown. The number of cell clones with those deletions are shown in the first column. In total, we analyzed 22 sequences, the total number of clones in which a cassette was present is shown at the bottom of the deletion columns.

indeed not observed. Deletion of C requires recombination at the H1 promoter, deletion of A is possible only through recombination at the terminator sequence. In total, deletion of A (4x) is seen less frequently than deletion of C (6x), which indicates that recombination at the H1 promoter is more likely, as would be expected since it represents the largest repeat sequence. Deletion of B occurs most often, in 16 of the 22 clones analyzed. We also observed deletions in cell clones transduced with the shRNA2 lentiviral vector (data not shown). Since deletion of each expression cassette was frequently observed in the cell clones, these results explain why the magnitude of inhibition was reduced for

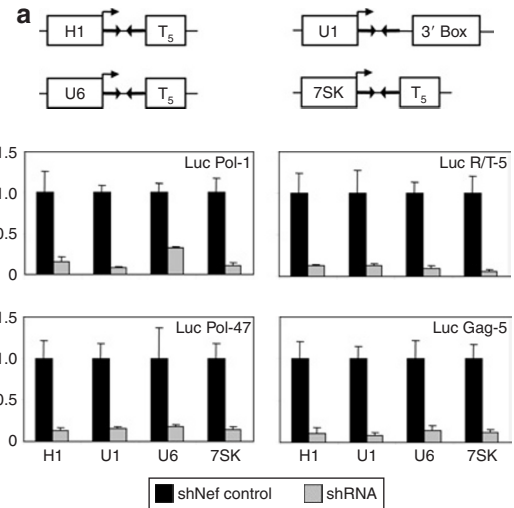


Figure 3 Similar levels of inhibition with different promoters and short hairpin RNA (shRNAs). **(a)** Different promoters used for shRNA expression. Three human polymerase III promoters, H1, U6, and 7SK, and the U1 human polymerase II promoter were used for shRNA expression. All expression cassettes have well-defined transcription start and termination sites. **(b)** Luciferase reporters for the conserved target sequences Gag-5, Pol-1, Pol-47, and R/T-5 were co-transfected with the control shNef expressing vector (black bars) or the specific shRNA vector (gray bars). Averages and SDs represent three independent transfections, the average activity of the shNef control for each promoter was set at 1.0. RLU, relative light units.

all shRNAs in cells transduced with shRNA2 and shRNA3. The same deletions with shRNA2 or shRNA3 have also been seen in a transduced T-cell line, SupT1, with 6 of 11 (55%) and 2 of 15 (13%) cell clones containing an intact double or triple cassette, respectively. Thus, repeat sequences should be avoided for effective multiple shRNA expression from a lentiviral vector.

Different promoters to drive shRNA expression

In order to prevent recombination, we tested four different promoters for shRNA expression (**Figure 3a**): the human H1, 7SK, and U6 polymerase III promoters^{4,31,32} and the human U1 polymerase II promoter.³³ These genetic elements do not have homologous sequences and were selected because the transcription start and termination sites are well defined, thus producing a discrete shRNA-inhibitor molecule. For each promoter, we made five different shRNA expression constructs against Nef¹, Gag-5, Pol-1, Pol-47, and R/T-5 sequences (**Figure 1a**). In co-transfection experiments, we compared the activity of each expression cassette against the appropriate reporter (**Figure 3b**). For each promoter-reporter combination, we used the corresponding shNef construct as a control for which the activity was set at 1.0. The Pol-1 shRNA made from the four promoters similarly reduced expression of the Luc Pol-1 reporter (top left panel in **Figure 3b**). Very similar results were obtained for the other three hairpins in combination with their respective reporter.

Effective multiple shRNA expression from different promoters

Since Luciferase knock-down was similar for all combinations of shRNAs and promoters, we made an arbitrary selection of four

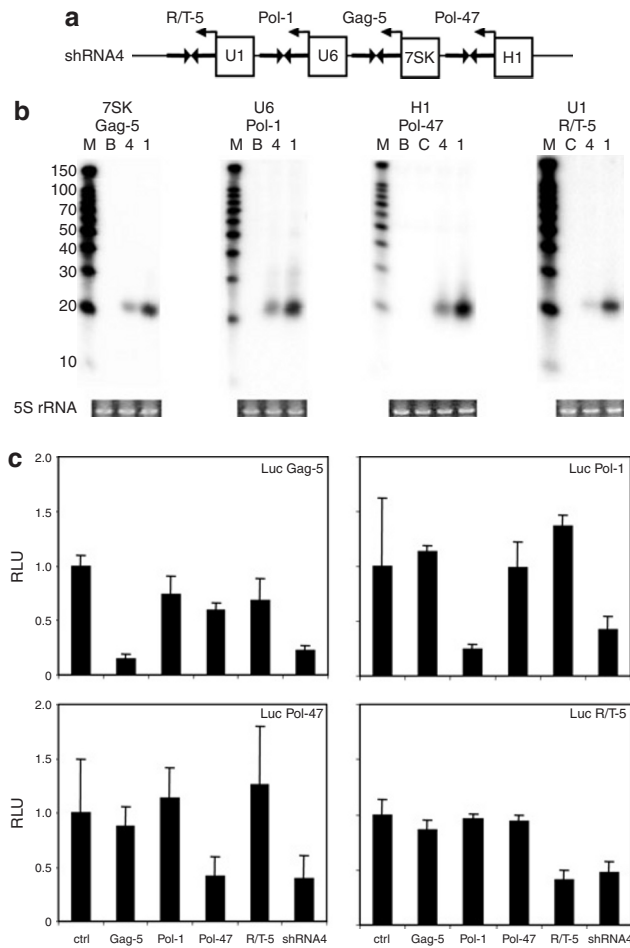


Figure 4 Single or multiple short hairpin RNA (shRNA) expression results in similar inhibition per shRNA. **(a)** Multiple shRNA expression construct. From a single construct, four different shRNAs (Pol-47, Gag-5, Pol-1, and R/T-5) were expressed from a human polymerase III promoter: H1, U6, and 7SK, and the U1 human polymerase II promoter, respectively. **(b)** Detection of small interfering RNAs expressed from shRNA4 (4) and the single shRNAs expressed from the corresponding promoters (1). As controls, a pBS (B) or a control shRNA construct (C) was used. Total RNA was isolated from transfected 293T cells and used for Northern blotting. 5S ribosomal RNA (rRNA) was used as loading control. M, size markers. **(c)** The indicated Luciferase reporters were co-transfected with the control shNef expressing vector (ctrl), the indicated single shRNA expression vector and the shRNA4 vector. Averages and SDs represent three independent transfections, the average activity of the shNef control was set at 1.0.

promoter-shRNAs for our combinatorial approach. The shRNA4 combination was made with the H1 Pol-47, 7SK Gag-5, U6 Pol-1, and U1 R/T-5 cassettes (Figure 4a). We first confirmed siRNA expression by detection of siRNAs with a Northern blot. All siRNAs were expressed from the shRNA4 construct, albeit somewhat reduced when compared to the corresponding single shRNA-expression vector (Figure 4b). This may be caused by the transfection of a relatively high amount of vector construct (5 µg), which may restrict optimal shRNA processing by limitation of the cellular components of the RNAi machinery.³⁴ In a more quantitative setting, we avoid such a competition effect by transfecting a low amount of vector construct (2 ng). We tested the shRNA4 construct in co-transfections with the four Luciferase reporters

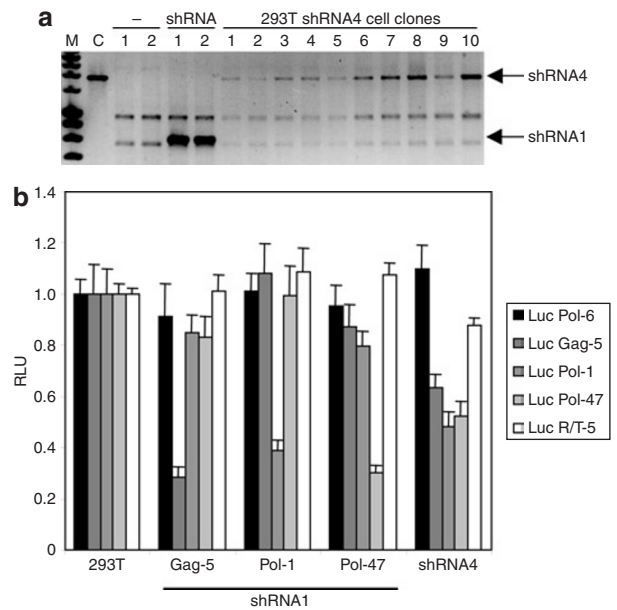


Figure 5 The shRNA4 cassette is intact in transduced cells. **(a)** 293T cells were transduced with the shRNA4 lentiviral vector at low multiplicity of infection (0.15) and cell clones were selected. A polymerase chain reaction was performed on genomic DNA of 10 selected cell clones. Bands corresponding to the quadruple cassette were observed for all cell clones. As controls, the shRNA4 plasmid and genomic DNA obtained from untransduced 293T (-) and shRNA1-transduced cell clones were included. **(b)** 293T cells transduced with the shRNA4 vector and green fluorescent protein positive cells were selected with live fluorescence-activated cell sorting. Cells were transfected with the indicated reporters. Controls were included, untransduced cells (293T) and the previously made shRNA1-transduced stable cell lines. Averages and SDs represent four independent transfections, the average activity of the 293T control was set at 1.0 for each reporter. shRNA, short hairpin RNA.

(Figure 4c). Control cells received no shRNA inhibitor (ctrl, activity set at 1.0). The shRNA4 construct was active against all four reporters. In fact, the inhibitory potential was similar to that of the appropriate single shRNA constructs. These results confirm that multiple shRNAs can be efficiently expressed from a single construct using different promoters.

Lentiviral vectors that stably express multiple shRNAs

We inserted the shRNA4 expression cassette (Figure 4a) in the lentiviral vector JS1 (Figure 1b). We produced lentiviral vector and transduced 293T cells at low MOI (0.15) and selected GFP-positive cells with live FACS. First, we selected 10 transduced cell clones to check the status of the integrated provirus. We performed the PCR across the insert and observed only the band corresponding to the size of the shRNA4 cassette (Figure 5a). The control lane (C) shows a PCR on the input shRNA4 plasmid. Two-cell clones, of either untransduced or Pol-47 transduced (shRNA1) cells, are also shown as controls. Thus, the shRNA4 cassette with the four different promoters is stably transduced. Second, we compared Luciferase reporter inhibition in the transduced 293T cells with the previously constructed single shRNA 293T cell lines (Figure 5b). Note that these shRNA1 cell lines express the indicated shRNA from the H1 promoter. Reporter inhibition in shRNA1 cells is similar in magnitude for each shRNA, and fully specific for the matching reporter. In the shRNA4 cells, inhibition

is observed with all four reporters. Quantitatively, a similar level of reporter inhibition is observed for Gag-5, Pol-1, and Pol-47. R/T-5, expressed in shRNA4 from the U1 polymerase II promoter, has a reduced magnitude of inhibition as compared to the other shRNAs, which coincides with a reduced expression level (Figure 4b). Combined, these data show that recombination was prevented by using different promoters to drive shRNA expression and that we now have four different shRNAs simultaneously expressed from a single lentiviral vector.

Prevention of HIV-1 escape by simultaneous expression of four shRNAs

To test the effect of multiple shRNA expression on HIV-1 replication, we stably transduced the SupT1 T-cell line that is fully susceptible for replication of the CXCR4-using LAI isolate. We produced the lentiviral vectors JS1, Pol-47, and shRNA4 with a codon-optimized Gag-Pol packaging construct to avoid targeting of viral sequences in the vector system, which results in

Table 1 Lentiviral vector titers of supernatant on SupT1 cells (N = 3)

Vector	Transducing units/ml
JS1	$1.1 \times 10^7 \pm 3.9 \times 10^6$
shRNA1 (Pol-47)	$5.1 \times 10^6 \pm 1.4 \times 10^6$
shRNA4	$6.1 \times 10^5 \pm 1.1 \times 10^5$

Abbreviations: shRNA, short hairpin RNA.

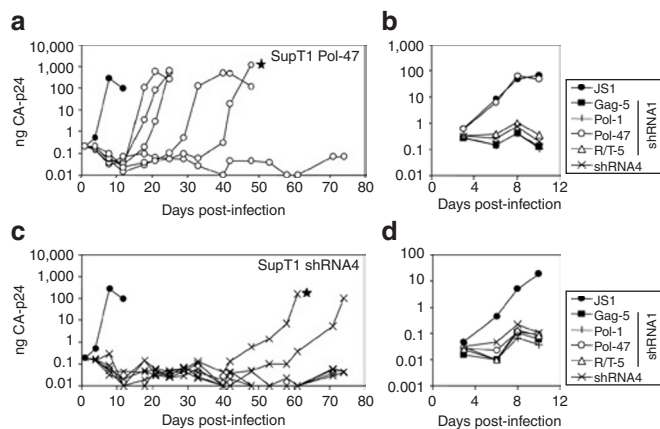


Figure 6 Prevention of escape with four short hairpin RNAs (shRNAs). (a) Six Pol-47 transduced SupT1 cell lines (circle) were infected with human immunodeficiency virus type 1 (HIV-1) and virus replication was monitored by measuring CA-p24 for up to 75 days. A control vector-transduced cell line was included (closed circles). In five of six cultures, HIV-1 started to replicate. (b) This replicating virus showed a resistant phenotype to the shRNA against Pol-47. For example, the replicating virus (marked with a star in a) was cell-free passaged on a control cell line (JS1), shRNA1 cell lines (Gag-5, Pol-1, Pol-47, and R/T-5) and the shRNA4 cell line. The virus replicated only on the control cell line and the Pol-47 cell line. (c) Six shRNA4-transduced SupT1 cell lines were infected with HIV-1 and virus replication was monitored for up to 75 days. A control vector-transduced cell line was included (closed circles). Initial virus replication was potently inhibited. After 40 days slow virus spread was scored in two cultures. (d) Cell-free virus (marked with a star in c) was used to infect a control cell line (JS1), shRNA1 cell lines (Gag-5, Pol-1, Pol-47, and R/T-5) and the shRNA4 cell line. The virus could replicate exclusively in the control cell line, indicating that the virus emerging in c is not resistant. The same result was obtained for the other positive culture.

reduced vector titers.^{24,35} The titer of the shRNA4 vector was reduced 18-fold as compared to the JS1 vector (Table 1), which is likely due to the insertion of the relatively large quadruple cassette (1,492 nt), as increased vector genome size is known to have a negative effect on titer.³⁶ Concentrated lentiviral vector stocks with similar titers were used to transduce cells at a low MOI (0.15) and transduced cells were selected by FACS for GFP-expression. All cell lines were viable and no differences in growth rates were observed. We infected six independent cultures of each cell line with the HIV-1 LAI isolate.³⁷ Efficient replication of this virus was measured in control vector-transduced cells (Figure 6a). Virus replication was initially potently inhibited in Pol-47 cells (Figure 6a). However, after extended culturing, the replicating virus emerged in five of six cultures as judged by an increase in CA-p24 in the culture medium and syncytia formation. We tested this putative escape virus by infection of a set of SupT1-transduced cells. This virus indeed showed a resistant phenotype, as it only replicated in the control cells and Pol-47 cells, but not in cells that express another shRNA inhibitor (Gag-5, Pol-1, or R/T-5) or the shRNA4 combination (Figure 6b). Sequence analysis confirmed resistance, as point mutations within the 19-nucleotide Pol-47 target sequence of the five escape cultures were observed (G8A, 2 × G15A, G9A, and G12T).

Simultaneously we tested HIV-1 replication in the shRNA4 cell line. Prolonged inhibition of HIV-1 replication was observed up to 75 days in four of six cultures (Figure 6c). Inhibition of virus replication was initially potent; however, slowly replicating virus did emerge in two cultures after 40 days. These viruses could represent escape viruses, perhaps still partially inhibited or slowly replicating due to mutations in multiple highly conserved sequences, resulting in a loss of viral fitness. Alternatively, slow spreading of the input wild-type virus may occur when it is not blocked completely. We and others have described such a pseudo-escape phenomenon in cell lines that allow efficient viral spread via cell-cell contact.^{11,38} To critically discriminate between these possibilities, we passaged the accumulated virus to a set of uninfected cells. The virus was able to replicate in the control SupT1 cells (JS1), but not in any of the shRNA-expressing cells, with either a single inhibitor (Gag-5, Pol-1, Pol-47, and R/T-5) or shRNA4 (Figure 6d). This result indicates that the breakthrough virus is not resistant to any

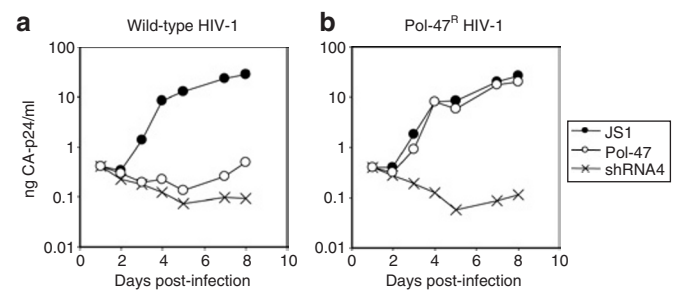


Figure 7 Potent inhibition of wild-type and Pol-47 resistant human immunodeficiency virus type 1 (HIV-1) in shRNA4-transduced primary CD4⁺ cells. Primary CD4⁺ cells were transduced with JS1 (closed circle), Pol-47 (open circle) and shRNA4 (cross) lentiviral vectors at low multiplicity of infection and green fluorescent protein positive cells selected with live fluorescence-activated cell sorting. Transduced cells were infected with (a) the wild-type virus or (b) a Pol-47 resistant virus. Virus replication was monitored by measuring CA-p24.

of the four shRNAs expressed in the shRNA4 cell line. This important conclusion was confirmed by sequencing of all four target sequences in the viral genome. Not a single point mutation was observed in any of the four targets.

Potent inhibition of an escape virus in primary CD4⁺ cells by four shRNAs

CD4⁺ T cells constitute the major cell population implicated in HIV-1 infection and making these cells resistant to HIV-1 is a key aspect of anti-HIV gene therapy. Thus, we also tested our lentiviral vectors in transduced primary CD4⁺ T cells. Again, cells were transduced at low MOI (0.2) with the control vector, JS1, shRNA1 vector Pol-47, or the shRNA4 vector and transduced cells were selected. As with the transduced SupT1 cell lines, no differences in viability or growth rates were observed. Next, we challenged the transduced cells with the wild-type HIV-1 virus (Figure 7a). In the control cells, efficient virus replication was detected. By contrast, virus replication was strongly inhibited in both the Pol-47 and shRNA4 cells, but more potent inhibition was scored in the latter cells. We also infected the transduced cells with an HIV-1 mutant that is resistant to Pol-47 due to a G to A mutation at target position 8 and 15 (Figure 7b). The mutant virus replicated equally well in the control JS-1 and Pol-47 cells, confirming its resistance to Pol-47, but it was as strongly inhibited in the shRNA4 cell line as the wild-type virus. These results show that the combined action of the four shRNAs results in a more potent inhibition as compared to a single shRNA in primary CD4⁺ cells.

DISCUSSION

For the development of an effective RNAi-based gene therapy against HIV-1, simultaneous expression of multiple shRNAs is required to avoid viral escape. A relatively simple strategy would be to express each shRNA from a separate promoter. Several groups have reported expression of multiple RNAs from separate promoters in a lentiviral vector.^{6,9,19,25,39} Here we performed an in-depth analysis of a multiple shRNA expression strategy in which three shRNAs are expressed from repeated promoters. We found that this expression strategy causes frequent recombination within the lentiviral vector genome at repeat sequences of the expression cassette, resulting in deletion of one or two cassettes. Thus, when cells are transduced with the shRNA3 lentiviral vector, the result is a heterogeneous population of integrated lentivirus proviral DNA expressing one, two, or three shRNAs. For the development of a successful RNAi-based gene therapy approach against HIV-1, this multiple shRNA expression strategy is unlikely to be successful. Cell populations that express only a single shRNA will provide a niche in which resistant viruses will be selected, ultimately leading to full resistance to all shRNAs.

In order to prevent recombination we selected four different promoters that lack sequence similarity for our multiple shRNA expression strategy. The human polymerase III promoters H1, U6, and 7SK and the U1 human polymerase II promoters^{4,31-33} were selected because they have well defined transcription start and termination sites. Differences in inhibitory activity should therefore reflect different expression levels. Activities of these promoters

have been compared before, but only with a subset of two promoters.^{32,33,40,41} We now tested and compared four promoters and measured similar shRNA inhibitory activity for each of these promoters. Also, in a combined construct, each expression cassette was active, confirming that multiple shRNA expression from these four promoters is a valid approach.

We constructed a lentiviral vector with the shRNA4 design. Analysis of transduced cell clones indicated that this vector does not recombine. The shRNA4 vector titer was reduced due to an increase in vector genome size, but we note that the PGK promoter and GFP gene (1,255 nt) will eventually be removed from the clinical vector, thus avoiding this production problem. We next tested the effect on HIV-1 replication in a gene therapy setting. In a transduced cell line expressing only a single shRNA, HIV-1 could escape by mutating the target sequence. By contrast, HIV-1 did not escape in the shRNA4-transduced cell line. However, viral spread was observed in two of the six infected shRNA4 cell cultures, though these viruses are not true escape mutants, since they did not have a resistant phenotype upon viral passage to fresh cells. Furthermore, no genotypic changes were observed in the four target sequences. This result indicates that virus replication is not completely blocked by the combined expression of four shRNAs. This should not come as a surprise since RNAi does not target the incoming genome^{42,43} and RNAi does not silence gene expression completely. Thus, virus replication may still occur, albeit at a very low level, perhaps slowly accumulating until a certain threshold is reached at which CA-p24 production can be measured. Most importantly, we did not witness viral escape, which shows that the imposed genetic barrier is too high for the virus to overcome inhibition by four shRNAs.^{1,12,13}

It is difficult to assess the implications of our results for gene therapy in which T cells or blood stem cells of HIV-1-infected patients are treated *ex vivo*. With only a single lentiviral vector copy per cell, we obtained durable inhibition for up to 40 days with the shRNA4 vector. In addition, we also observed that primary CD4⁺ T cells transduced with the shRNA4 lentivirus are viable and have normal growth rates and we have shown that wild-type HIV-1 or a Pol-47-resistant virus is potently inhibited in these cells. The next step in the evaluation of the therapeutic potential of the shRNA4 vector is testing in a relevant *in vivo* system. Recently, a mouse model with a humanized immune system was developed that we propose to use for this purpose.^{22,44} We will study the safety of the shRNA4 vector by analyzing the development of the immune system from transduced human CD34⁺ precursor cells. In addition, we can also infect these mice with HIV-1 to evaluate the *in vivo* efficacy and durability of the multiple shRNA approach.^{45,46}

Gene therapy to protect cells against HIV-1 holds great promise in the treatment of HIV-1-infected individuals.^{47,48} Recently, the first phase I clinical trial involving lentiviral vectors was shown to be safe.⁴⁹ In this clinical trial, HIV-1 patients were treated with a conditionally replicating viral vector encoding an antisense sequence directed against the HIV-1 genome. This year, a clinical trial is expected to start which combines different anti-HIV RNAs in a single lentiviral vector.²⁵ The promising results presented here strongly support the development of our multiple shRNA strategy

against HIV-1 that, when shown to be safe and effective *in vivo*, may be next in line for clinical testing.

MATERIALS AND METHODS

Plasmid construction. JS1 (pRRLLcpptpgkfpfpreSsin),⁵⁰ the single (shRNA1), double (shRNA2), and triple (shRNA3) shRNA lentiviral vectors⁹ were previously described. Constructs used for shRNA expression are based on pSUPER (OligoEngine, Seattle, WA), pSilencer 2.0-U6 (Ambion, Austin, TX), psiRNA-h7Skhygro G1 (Invivogen, San Diego, CA), and pGeneClip-BasicVector (Promega, Madison, WI), which contain the human H1, U6, 7SK polymerase III promoters, and human U1 polymerase II promoter, respectively (which we renamed pH1, pU6, p7SK, and pU1). First, shRNA expression plasmids were constructed by inserting annealed oligonucleotides, encoding the shRNA transcript, into the appropriate insertion sites. In our oligonucleotide design additional restriction sites were added at 3' of the transcription termination sites of the U6 and 7SK constructs to facilitate cloning of the shRNA4 construct (*Bgl*II, *Zra*I, *Cla*I, *Xho*I for U6 and *Sal*I, *Xho*I for 7SK). The shRNA4 plasmid was constructed from the different shRNA expression plasmids (pH1Pol47, p7SKGag5, pU6Pol1, and pU1R/T5) as follows. First, the 7SK cassette was inserted behind the H1 cassette with the *Cla*I/*Sal*I restriction sites. Second, the U6 cassette was excised with *Eco*RI/*Bgl*II and inserted in front of the U1 promoter using the *Eco*RI/*Bam*HI sites. Finally, the U6-U1 double cassette was inserted in the *Sal*I/*Xho*I site of the H1-7SK construct, yielding pH1Pol47-7SKGag5-U6Pol1-U1R/T5, or shRNA4. The shRNA4 cassette was excised with *Sma*I/*Xho*I and inserted in the lentiviral vector backbone of JS1 in the multiple cloning site (*Eco*RV/*Xho*I), resulting in JS1-shRNA4.

Cell culture. Human embryonic kidney 293T adherent cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) and SupT1 suspension cells were grown in Advanced Rosewell Park Memorial Institute medium (Invitrogen, Carlsbad, CA) supplemented with L-glutamine, 1% fetal calf serum, penicillin (30 U/ml) and streptomycin (30 µg/ml), both in a humidified chamber at 37°C and 5% CO₂. Peripheral blood mononuclear cells were grown in Rosewell Park Memorial Institute medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) and stimulated with interleukin-2 (100 U/ml) and phytohemagglutinin (4 µg/ml) for 2 days after which CD8 cells were depleted, cells were subsequently cultured without phytohemagglutinin.

Lentiviral vector production. The lentiviral vector was produced as previously described.^{9,24} Briefly, the vector was produced by co-transfection of lentiviral vector plasmid and packaging plasmids pSYNGP, pRSV-rev, and pVSV-g with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After transfection, the medium was replaced with OptiMEM (Invitrogen, Carlsbad, CA). The lentiviral vector containing supernatant was collected, filtered (0.45 µm), and concentrated using an Amicon Ultra concentrator, MWCO 100,000 (Millipore, Bedford, MA) and aliquots were stored at -80°C.

Lentiviral vector transduction. Lentiviral vector stocks were titrated on SupT1 cells, 293T cells and CD4⁺ primary cells. 293T cells and SupT1 cells were transduced with the indicated MOI. Peripheral blood mononuclear cells were transduced after CD8 depletion. Two to three days after transduction, live cells were sorted with FACS and GFP-positive cells were selected. For selection of transduced cell clones, single GFP-positive cells were selected with FACS.

Transfection experiments. Co-transfection of firefly Luciferase reporters and the shRNA-expression plasmids or transfections of firefly Luciferase reporters in the stable cell lines were performed as previously described,⁹ with an internal control Renilla Luciferase, pRL. Briefly, 100 ng of firefly reporter was co-transfected with 20 ng (Figures 1c and 3b) or 2 ng

(Figure 4c) of pSUPER single shRNA constructs and equimolar quantities of the other constructs, with 2 ng of the internal control pRL, in a 24-wells format. Firefly and Renilla Luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

HIV-1 infection. HIV-1 LAI and the Pol-47 resistant virus was produced in transfected 293T cells. Virus production was measured by CA-p24 enzyme-linked immunosorbent assay. SupT1 cells (5-ml cultures, 2.5 × 10⁶ cells) were infected with 1 ng of CA-p24. Transduced CD4⁺ cells (0.2 ml, 1 × 10⁵ cells) were infected with 0.2 ng of CA-p24. Virus spread was monitored by measuring CA-p24 production.

PCR and sequencing. Cellular DNA was extracted from cell clones (5 × 10⁶ cells) with the Qiagen DNeasy kit (Qiagen, Valencia, CA). PCR was performed across the multiple cloning site with the primer pair fw1 (AGTGAACGGATCTCGACGGTAT) and rev1 (TAGTAACGGCCGCC AGTGTGCT), with 30 cycles (1-minute denaturation at 94°C, 30 seconds annealing at 62°C and 3-minute extension at 72°C). The PCR products were used for standard direct sequencing with the Big Dye Terminator Cycle Sequencing kit (ABI, Foster City, CA) using the same primers, 1 mol/l betaine was added to the sequence reaction.

siRNA detection by Northern blotting. For transfection, human embryonic kidney 293T cells were plated in 6-well plates at a density of 750,000 cells per well in 3 ml Dulbecco's Modified Eagle's Medium with fetal calf serum without antibiotics. The next day, equimolar quantities of shRNA constructs were transfected (e.g., 5 µg pSUPER) using Lipofectamine 2000. Total RNA was purified using the mirVana miRNA isolation kit (Ambion, Austin, TX). For Northern blot analysis, 15 µg total RNA was heated for 5 minutes at 95°C and electrophoresed in a 15% denaturing polyacrylamide gel (pre-cast Novex TBU gel, Invitrogen, Carlsbad, CA). To check for equal loading, the gel was stained with 2 µg/ml ethidium bromide in ultrapure water for 20 minutes. Destaining was performed by rinsing the gel three times with ultrapure water for 10 minutes. The bands were visualized under ultraviolet light. Then the RNAs in the gel were electro-transferred to a positively charged nylon membrane (Boehringer Mannheim, GmbH, Mannheim, Germany). The RNA was cross-linked to the membrane using ultraviolet light at a wavelength of 254 nm (1,200 µJ × 100). Locked nucleic acid oligonucleotides were 5'-end labeled with the kinaseMax kit (Ambion, Austin, TX) in the presence of 1 µl of [γ -³²P]ATP (0.37 mol/lBq/µl Amersham Biosciences, Piscataway, NJ) and used as probes. We used the following locked nucleic acid oligonucleotides: 5'-ATGGCAGGAAGAAGCGGAG-3' (siRNA R/T 5), 5'-ACAGGAGCAGATGATACAG-3' (siRNA pol 1), 5'-GAAGAAATGATGACAGCAT-3' (siRNAs gag), and 5'-GTGAAGG GGCAGTAGTAAT-3' (siRNAs pol 47). Underlined are the positions that contain locked nucleic acids. To remove unincorporated labeled nucleotides, the probes were purified using the Sephadex G-25 spin columns (Amersham Biosciences, Piscataway, NJ) according to manufacturer's protocol. *In situ* hybridizations were performed at 42°C with labeled locked nucleic acid oligonucleotides in 10 ml ULTRAhyb hybridization buffer (Ambion, Austin, TX) according to the manufacturer's instructions. After hybridization, the membranes were washed twice for 5 minutes at 42°C in 2 × 0.3 mol/l sodium chloride, 0.03 sodium citrate pH 7.3/0.1% sodium dodecyl sulfate and 2 × 15 minutes in 0.1 × 0.3 mol/l sodium chloride, 0.03 sodium citrate, pH 7.3/0.1% sodium dodecyl sulfate at 42°C and signals were detected by autoradiography using a phosphorimager.

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