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Activity of Tar in Inducible inhibition of HIV replication by foamy virus vector expressing siRNAs under the control of HIV LTR

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Abstract

In this report we describe foamy virus vectors with conditional expression of short interfering RNAs (siRNAs) in HIV infected cells. Short hairpin RNAs (shRNAs) based on two targets in the 5' end of the untranslated region and one in the rev gene flanked with 5' and 3' micro RNA 30 (miR30) sequences were synthesized and placed under the control of an HIV promoter for Tat mediated expression. HIV permissive cells were transduced with foamy virus vectors containing each hybrid shRNA expression cassette and tested for their efficacy on the inhibition of HIV replication. Effective Tat dependent expression of the shRNAs, as well as GFP placed downstream each shRNA was evident. In addition the results show inhibition of HIV replication by greater than 98%. Interestingly, transduction of cells with a vector lacking an shRNA also revealed GFP expression in the presence of Tat with similar levels of inhibition of virus replication. When the TAR region was removed from this vector there was neither reduction in virus replication nor Tat induced GFP expression. These results suggest that TAR in the vector, which Tat interacts to promote expression of the shRNA, is a potent inhibitor of virus replication. Previous studies with TAR regulated expression of antiviral genes ignore the contribution of TAR in the repression of virus replication. Interpretation of effective inhibition of HIV replication by antiviral genes located downstream of TAR while neglecting the efficacy of a potent repression by TAR is misleading.

Keywords

Foamy virus vector; HIV gene therapy; miRNA; TAR

Introduction

Gene therapy delivering short hairpin RNA (shRNA) into target cells to inhibit virus replication through the RNA interference mechanism has been widely reported (reviewed in Singh, 2008). RNA interference (RNAi) is a conserved gene silencing mechanism that recognizes double-stranded RNA as a signal to trigger the sequence specific degradation of homologous mRNA (reviewed in (Hannon, 2002; Sharp, 2001)). The double strand serves as an initial trigger of RNA silencing, and after recognition, is processed by RNase into short fragments of 21 nucleotides. These short interfering RNAs (siRNAs) are then incorporated into a dsRNA-induced silencing complex (RISC) to guide cycles of specific RNA degradation. A key finding is the generation of small dsRNAs (21-25 nucleotides) from input dsRNA during post-

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transcriptional gene silencing and RNAi (Hamilton and Baulcombe, 1999; Hammond *et al.*, 2000; Parrish *et al.*, 2000; Yang, Lu, and Erickson, 2000; Zamore *et al.*, 2000). These observations open a path toward the use of siRNAs in reverse genetics and therapeutic tool. To these ends a hairpin type siRNA (shRNA) can be expressed and delivered to target cells via viral vector. siRNAs elicit potent targeted degradation of complementary RNA sequences and are effective at low concentrations. Specific inhibition of gene expression by introducing siRNAs into a cell culture system has been accomplished (Elbashir *et al.*, 2001; Paddison *et al.*, 2002; Paddison, Caudy, and Hannon, 2002). Furthermore, siRNAs are shown to be more effective than the antisense/ribozyme strategy implicating that siRNAs are a potent gene therapy strategy against HIV infection (Lee *et al.*, 2002; Novina *et al.*, 2002).

Initially, this gene silencing via transcript degradation was believed to be exquisitely specific requiring near-identity between the siRNA and the target mRNA. However, subsequent reports have raised concerns regarding the clarity of siRNA specificity and processing that may produce differential effects revealing a multitude of off-target effects (Bridge *et al.*, 2003; Jackson *et al.*, 2003; Lin *et al.*, 2005; Saxena, Jonsson, and Dutta, 2003; Scacheri *et al.*, 2004; Sledz *et al.*, 2003). The off-target effects, therefore, require complete understanding in order to improve the design of more specific siRNAs. In the meantime, caution is warranted in interpreting gene function and phenotypes resulting from RNAi experiments. Control experiments that check for off-target effects should also be included for each siRNA. Although the off-target effects can potentially become a major obstacle for siRNA based therapeutics, several reports indicate that the off-target effects appear to occur only at high concentrations of siRNA (Chi *et al.*, 2003; Persengiev, Zhu, and Green, 2004; Semizarov *et al.*, 2003).

RNAi under the control of pol III are expressed constitutively in all cell types directing high levels of shRNA expression. Pol III promoter based expression systems have no cell specificity and exceedingly high levels of shRNA expression will increase the chances of off-target silencing (Chi *et al.*, 2003; Jackson and Linsley, 2004; Lin *et al.*, 2005; Persengiev, Zhu, and Green, 2004; Semizarov *et al.*, 2003). Therapies based on siRNA would benefit with the ability to regulate shRNA expression in virally infected cells, which subsequently limits potential off-target effects. An RNA Pol II promoter will be advantageous for cell or tissue specific expression of shRNA (Malphettes and Fussenegger, 2004; Wiznerowicz and Trono, 2003; Zhou, Xia, and Xu, 2005). Effective expression of shRNA can be accomplished by taking advantage of the micro RNA (miRNA) expression system, which is regulated by the RNA pol II promoter (Malphettes and Fussenegger, 2004; Wiznerowicz and Trono, 2003; Zhou, Xia, and Xu, 2005). Such an approach has widely been used by flanking shRNA with 5' and 3' miRNA 30 (miR30) sequences (Zeng and Cullen, 2005; Zhou, Xia, and Xu, 2005). Therefore, based on these findings inducible and regulated expression of shRNA can be developed which would limit potential cell toxicity while effectively targeting a specific gene of interest.

Limiting the expression of siRNAs in HIV infected cells could avoid off-target effects, which is a prevailing concern of siRNA therapeutic applications. To restrict siRNA expression in HIV infected cells efficient regulated expression of siRNA can be achieved by using an HIV-1 Tat dependent HIV promoter containing the TAR region (Unwalla *et al.*, 2006; Unwalla *et al.*, 2004). However, such approach with Tat regulated antiviral gene expression does not consider the contribution of TAR in the inhibition of viral replication from the vector in trans and assumes inhibition of HIV replication is only due to the antiviral element placed down stream of TAR. Furthermore, in those studies an HIV vector was used to deliver antiviral molecules to inhibit HIV replication, and within HIV infected individuals vector mobilization by HIV would be a major safety concern. Therefore, for HIV gene therapy an alternative vector that is safe and has no chance of vector mobilization by HIV is necessary. Foamy virus vectors fit such criteria and are ideal for HIV gene therapy since there is no vector mobilization by HIV infection. We synthesized shRNAs that target the rev gene and two regions in the long terminal

repeat (LTR) of HIV by flanking them with 5' and 3' region miR30 sequences under the control of the HIV LTR promoter for Tat regulated expression. The hybrid shRNAs were first placed in a foamy vector, and then tested for conditional expression and inhibition of HIV replication. Our results show the TAR, which allows the conditional expression of the shRNAs in the presence of Tat or HIV show potent inhibition of HIV replication to the level that it overshadows the demonstration of efficacy of the shRNAs in such TAR-Tat regulated expression systems.

Results

Inhibition of HIV-1 replication by targeted 5'-untranslated region (UTR) siRNA in 293T cells

Short hairpin siRNAs (shRNA) were designed based on 2 target regions in the LTR and rev of HIV-1. To initially evaluate the efficiency of the siRNAs, prior to testing in the context of miRNA, each shRNA was placed under the control of a U6 promoter for expression (Figure 1A) and tested for their ability to inhibit HIV replication. They were then compared to a previously described efficient siRNA that targets the rev gene (R) (Lee *et al.*, 2003; Li *et al.*, 2003). The expression of siRNA under the control of pU6 was confirmed by Northern blot analysis (Figure 1B). As shown in Figure 1, fully processed siRNA and the precursor shRNA were detected by Northern blots. To determine the efficacy on HIV replication the HIV provirus pNL4-3 was co-transfected with each shRNA-expressing construct into 293T cells. Supernatants from the transfected cells were harvested every day for 4 days to determine the levels of virus production by monitoring for reverse transcriptase (RT) enzyme activity. Inhibition of HIV replication by pU6-L1 and pU6-L2 were reaching 76% and 85%, respectively (Figure 1B). These levels of inhibition of HIV replication are comparable to that of anti-rev siRNA (82%). The L1, L2 and R targets were selected for hybrid shRNA designing containing the miRNA30 flanking sequences and to determine inducible expression and subsequent repression of HIV replication.

Tat-inducible expression of shiRNA

We have constructed expression cassettes using the HIV promoter region for conditional expression of siRNAs in cells infected with HIV. These constructs, for inducible and regulated expression of shRNA, exploit the miRNA system in order to utilize the RNA pol II promoter. Artificial RNA can be designed and expressed based on the features of existing miRNA genes, such as the gene encoding human miR30 (Zeng and Cullen, 2005; Zhou, Xia, and Xu, 2005). miRNAs with each shRNA (L1, L2, or R) flanked by 5' and 3' region of miR30 were constructed and placed under the control of the HIV LTR promoter (designated HL-L1, HL-L2, or HL-R) for conditional expression. For efficient regulated expression, the HIV promoter TAR region is fused to a minimal heat shock promoter (Hsp) sequence as previously described (Unwalla *et al.*, 2004). The expression system uses transcriptional read through of a minimal polyadenylation signal from an RNA Pol II promoter for expression of GFP (Unwalla *et al.*, 2006). Tat interaction with TAR upstream of Hsp allows recruitment of P-TEFb, which subsequently phosphorylates RNA polymerase at Hsp's transcription initiation complex resulting in transcription at position +1 of the shRNA. Therefore, from this system both TAR as well as regulated shRNA and GFP expression occurs. Each of the hybrid shRNAs corresponding to L1, L2, or R were placed under control of the HIV promoter and TAR-Hsp fusion expression system as shown in Figure 2A. To determine regulated expression under the control of HIV promoter we monitored GFP expression in HeLa cells expressing Tat (Figure 2B) using a construct without an shRNA (HL). In HeLa or 293T lacking the Tat protein we observed very few cells expressing GFP. The LTR promoter in such a vector appears to have weak leakiness and the minimal poly A located upstream from GFP may have contributed to such weak basal activity. The Tat expressing HeLa or 293T cells showed significantly enhanced GFP expression. When TAR was removed (HLdTat) the level of GFP expression in Tat

expressing HeLa cells reduced to the expression level observed in HeLa cells lacking Tat, indicating a regulated expression system. In cells transfected with the Hsp minimal promoter region deletion (HLdHsp), however, the number of GFP expressing cells were equivalent to cells transfected with HL indicating that the removal of Hsp did not affect GFP expression. Conditional expression of the hybrid shRNAs was examined by Northern blot analysis. Each of the shRNA constructs were transfected alone or with a Tat expressing plasmid (pTATZ) into 293T cells. Small size RNA was isolated, blotted, and hybridized with radioactive $\gamma^{32}\text{P}$ ATP labeled specific probe. As shown in Figure 2C we can readily detect hybrid shRNA expression in the presence of Tat. In contrast no or minute amounts of shRNA was present in the absence of Tat confirming regulated expression of the shRNAs.

The antiviral activity of TAR expressed from these constructs was determined by co-transfecting the expression cassettes with the HIV (NL4-3) provirus DNA (Figure 3). We have also determined the efficacy of each shRNA on HIV replication along with TAR. In addition, the L1 and R shRNAs were placed in one expression vector (HL-L1R) to demonstrate antiviral activity with multiple targets. As shown in Figure 3, co-transfection of HL with HIV provirus revealed that the TAR was a potent inhibitor of HIV replication showing inhibition of virus replication by 86%. When TAR was deleted (HLdTAR) we observed no repression of HIV replication. Comparable levels of repression of HIV replication were observed with TAR plus L2 (HL-L2, 87%) and TAR plus R (HL-R, 89%). When the Hsp minimal promoter region was deleted (HLdHsp) instead of TAR HIV replication was inhibited by 76%, thus showing that the deletion of Hsp has only a minor effect in downstream miRNA expression. Transfection of constructs containing TAR plus L1 (HL-L1) showed inhibition of HIV replication reaching 98%. When more than one shRNA was used (HL-L1R) inhibition of virus replication was 98%. These levels of inhibition of virus replication are comparable to that of where no shRNA was placed downstream of TAR (HL), thus indicating that the actual activity of these shRNAs cannot be extrapolated from such a vector system by the present quick and short-term HIV replication assay. Furthermore, a majority of the antiviral activity could be due to TAR. The slight increase in the repression of virus replication with vectors HL-L1 and HL-L1R implicate a synergistic effect of TAR with L1 shRNA. However, with shRNA R, which the shRNA has been shown to be a potent inhibitor of HIV replication, we observed no significant difference when compared with that of TAR alone (HL). The L1 and R shRNAs may be processed efficiently and contribute significantly to inhibition of virus replication. However, since TAR is required for expression of these shRNAs free TAR is always available to influence HIV replication. Examining virus replication with TAR alone or plus the shRNAs over a long period (longer than 4 days) may help give insight about the synergistic effects of TAR and inducible shRNA expression on HIV replication.

Inhibition of HIV replication in cells transduced with SFVmac vector carrying siRNAs

Recombinant SFVmac vectors containing hybrid shRNAs with conditional expression were constructed (Figure 4A) in order to determine the efficacy of shRNAs delivered via foamy virus vector on HIV replication over a long period. Each of the SFVmac vectors containing the different shRNAs that target HIV were transduced along with packaging plasmids into 293T cells generating vector particles used to infect U87.CD4.CXCR4 cells (Björndal *et al.*, 1997), which are susceptible to HIV infection. Cells receiving no SFVmac vector were used as a control (mock). An additional control included SFVmac containing the expression cassette that was lacking TAR (SFV/HLdTAR). Conditional expression in transduced cells was monitored by Northern blot analysis for shRNA using specific probes (Figure 4C) and fluorescent cells for GFP (Figure 4B) in the presence or absence of Tat. The transduced cells were challenged with HIV at an moi of 0.005. Supernatants from the infected cells were harvested at intervals for 26 days to determine the levels of virus production by monitoring for capsid p24 protein by ELISA. As shown in Figure 5, HIV replication was detected 5 days post-

infection in the mock transduced cells, increasing exponentially thereafter until cell lysis began. Similar to the transient assay described above, HIV promoter driven expression of TAR alone showed potent inhibition of HIV replication at levels of more than 98% (days 9-22) in foamy vector transduced cells (SFV/HL). When siRNA that targets rev (SFV/HL-R) was included reduction of HIV replication was also more than 98% (days 9-22). In cells transduced with foamy vector, where TAR is deleted (SFV/HLdTAR), the HIV replication was comparable to mock transduced control cells. This confirms that in the absence of TAR there is no effective antiviral gene expression. To demonstrate the efficacy of the shRNAs that target rev or the LTR in the context of foamy virus vector while lacking TAR we placed an expression cassette for shRNAs R and L2 under the control of the cytomegalovirus (CMV) promoter (SFV/CMV-L2R) and tested the activity on HIV replication. We found that the inhibition of HIV replication to be similar (>98%) to that of cells transduced with foamy virus vector expressing TAR alone or shRNA R under the control of the HIV promoter. When TAR, shRNA R, and shRNA L2 (SFV/HL-L2R) were placed under the control of the HIV promoter in a foamy vector we observed similar optimum inhibition of HIV replication that is comparable to that of cells transduced with the foamy virus SFV/HL-R.

In order to examine whether there is a synergistic effect of TAR and shRNA we compared the inhibition of HIV replication in cells transduced with TAR alone (SFV/HL), TAR plus shRNAs (SFV/HL-R and SFV/HL-L2R), and CMV driven expression of the L2R shRNAs (SFV/CMV-L2R). As shown in Figure 6, a significantly higher repression of HIV replication ($p < 0.05$) was observed in SFV/HL-R transduced cells (days 12-26) compared to SFV/HL. Similarly, a higher level of inhibition was observed with SFV/HL-L2R vector than SFV/HL. While cells receiving the SFV/CMV-L2R vector (vector without TAR) showed a higher inhibition of HIV replication than cells transduced with SFV/HL but was less than cells receiving the SFV/HL-R vector (days 12-20). However, on days 22-26 the cells receiving SFV/CMV-L2R show a rise in HIV p24 as compared to SFV/HL, SFV/HL-R, and SFV/HL-L2R. The delay in HIV replication with in cells treated with SFV/CMV-L2R compared to SFV/HL suggests differences in the promoter activity or that L2R may be more effective than Tar. These results, first, suggest that TAR plays a key role for the optimum inhibition of virus replication, and secondly that there is a synergistic effect of TAR with shRNAs (either with single or double miRNAs). Although we observed a synergistic effect of the SFV/HL-R transduced cells as compared to those receiving the SFV/HL vector, inhibition of HIV replication was greater than 98% for both as compared to mock and SFV/HLdTAR transduced cells (Figure 5). Furthermore, during the first 9 days all interventions were effective at equivalent levels. The number of virus particles was low prior to day 9 and as result amount of Tat was also low. Therefore, low Tat and continuous expression of TAR from the vector could account for the effective inhibition in the first 9 days strengthening a role for Tar. These results taken together suggest that TAR is a potent inhibitor of HIV replication in vectors where other antiviral genes are regulated by the Tat-TAR interaction. Therefore, the efficacy of antiviral genes located downstream of TAR cannot be interpreted by neglecting the activity of TAR.

Discussion

In this study we have shown effective inhibition of HIV replication with conditional expressed siRNAs in viral infected cells that were delivered using a foamy virus vector. Therapies based on siRNA would benefit with the ability to regulate shRNA expression in virally infected cells, which subsequently limits potential off-target effects. An RNA Pol II promoter will be advantageous for cell or tissue specific expression of siRNA. The HIV promoter and TAR were used for regulated expression of shRNAs that target HIV noncoding regions and the rev gene. The use of HIV LTR promoter with TAR allows restricted expression of the antiviral siRNAs in virally infected cells. Efficient expression of these shRNAs in HIV infected cells were detected which led to effective inhibition of virus replication. We observed that TAR

expression alone from the HIV promoter inhibits HIV replication by greater than 98%. Previous reports using an HIV vector containing TAR regulated antiviral genes indicated effective inhibition of HIV replication (Unwalla *et al.*, 2006; Unwalla *et al.*, 2004). However, these studies did not examine the role of TAR, an integral part of the vector system, in which the Tat dependent expression of antiviral genes is located downstream. Our results show that TAR is a major contributor to the inhibition of virus replication in such conditional expression systems and that the expression of antiviral TAR is not restricted to infected cells, indicating that not all-antiviral gene expression in such a vector system is conditional.

The expression of antiviral shRNAs in these studies is Tat dependent and the effective inhibition of virus replication implicates no or minimal negative regulation of the HIV driven expression of the antiviral genes. As the data show HIV replication is not able to overcome the inhibitory effects of the siRNAs over the course of the experiment. Several studies show RNA based anti-HIV approaches such as ribozymes, shRNAs, and antisense RNA expressed from the HIV LTR to effectively inhibit viral replication in HIV infected cells (Dropulic, Hermankova, and Pitha, 1996; Paik *et al.*, 1997; Unwalla *et al.*, 2006; Unwalla *et al.*, 2004). These studies, however, also did not examine the role of TAR from the vector in HIV inhibition. Expression of TAR does not require Tat and thus its expression is not regulated. Our control where no shRNA is located downstream TAR (SFV/HL) effectively inhibited HIV replication. The inhibition levels were comparable with that of cells transduced with vector containing shRNAs in which their expression is dependent on TAR. In the SFV/HL control vector, Tat should help elongate the paused transcripts at TAR by interaction with TAR and host factors. This expression system uses the minimal Hsp promoter located downstream TAR. The minimal Hsp promoter allows the recruitment of P-TEFb that subsequently phosphorylates RNA polymerase at the Hsp transcription initiation complex resulting in transcriptional position +1 where GFP is placed. Therefore, the system permits regulated GFP expression only in the presence of Tat, which is confirmed in Figures 2 and 4. When TAR was removed there was neither GFP expression nor inhibition of HIV replication. Interestingly, when the minimal Hsp promoter region was removed we observed GFP expression in the presence of Tat and inhibition of virus replication that was comparable to the level seen for HL suggesting that the TAR region was important in reducing the HIV replication in such a vector system. Taken together these results implicate that Tat expressed from HIV does not bind all the TAR regions in the vector and as a result some free TAR RNA molecules are produced which subsequently inhibit virus replication. The system therefore allows TAR expression as well Tat regulated shRNA expression.

Significant inhibition of HIV replication in the absence of the hybrid shRNAs suggests the potency of TAR in reducing HIV replication. TAR has been used as a decoy to inhibit virus replication by competing with HIV for Tat binding (Barnor *et al.*, 2005; Li *et al.*, 2003; Lisiewicz *et al.*, 2000; Strayer *et al.*, 2002). In addition, recently it was reported that HIV TAR is processed by the Dicer enzyme to create a viral miRNA (Klase *et al.*, 2007; Ouellet *et al.*, 2008). TAR derived miRNA was shown to be involved in chromatin remodeling and a reduction in LTR driven gene expression (Klase *et al.*, 2007; Weinberg and Morris, 2006). Therefore, TAR derived miRNA can inhibit virus replication by regulating viral gene expression and probably repressing gene expression through transcriptional silencing (Klase *et al.*, 2007; Triboulet *et al.*, 2007; Weinberg and Morris, 2006). These findings of TAR miRNAs in infected cells and the observation that such repression of LTR driven expression implicates a possible mechanism for latent viral infections (Klase *et al.*, 2007; Triboulet *et al.*, 2007; Weinberg and Morris, 2006). Since miRNAs are in general effective at low concentration we speculate that the potent inhibition of virus replication we observed in SFV/HL vector transduced cells may have to do with TAR miRNA activity processed from the vector. The possible TAR miRNA generated from the SFV/HL vector may either be involved in cleaving Target viral mRNA or repression of viral gene expression. Furthermore, the potent

inhibition of virus replication in cells that received the SFV/HL vector implicates that the potential TAR miRNA may be very effective when supplied in trans.

Vectors based on lentiviruses such as HIV are promising since they can infect both dividing and nondividing cells (Akkina *et al.*, 1996; Naldini, 1998). A major concern with the usage of HIV vectors for HIV gene therapy is the possibility of vector mobilization during HIV replication. Enhanced vector mobilization could lead to adverse consequences, such as toxicity to individuals receiving gene therapy. An alternative effective vector for HIV gene therapy is crucial to overcome such potential problems. Foamy virus vectors can be an ideal vector system for HIV gene therapy. Inhibition of immunodeficiency virus replication has been demonstrated with antiviral genes delivered via foamy virus vectors (Park *et al.*, 2005; Taylor *et al.*, 2008). Here, we have shown Tat dependant expression of shRNAs that target HIV in virally infected cells transduced with foamy virus vector as well as effective reduction of HIV replication by TAR from the vector. Thus far no pathogenesis is associated with foamy virus infection. Integration analysis of foamy virus vector genome into host chromosome reveals a reduced risk for mutating or activating cellular genes when compared to lentiviruses, which corroborates the non-pathogenic phenotype of foamy virus infections (Hendrie *et al.*, 2008; Trobridge *et al.*, 2006). Foamy virus vectors transduce hematopoietic stem cells with high efficiency (Josephson *et al.*, 2002; Kiem *et al.*, 2007; Zucali *et al.*, 2002) and have recently been used to successfully cure a hematopoietic disease of dogs (Bauer *et al.*, 2008). These accomplishments will help develop ex vivo manipulation of hematopoietic stem cells and with subsequent transplantation of these modified cells in HIV infected individuals for regulated antiviral gene expression using a safe vector.

Materials and Methods

Cell cultures

The 293T cell line (human embryonic kidney fibroblasts) was obtained from American Type Culture Collection (Rockville, Md.). HeLa-tat-III and U87.CD4.CXCR4 cells were kindly provided by the NIH AIDS Research and Reference Reagent Program. The 293T and HeLa-tat-III cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with streptomycin (100 ug/ml), penicillin (100 ug/ml), and 10 % fetal calf serum. U87.CD4.CXCR4 cells were grown in DMEM containing 15% FBS plus 300 ug/ml geneticin and 1 ug/ml puromycin (Björndal *et al.*, 1997). Geneticin and Puromycin are used to select for stable CD4 and CXCR4 cells, respectively.

Construction of plasmids

Hairpin siRNAs that target rev or two different regions of the HIV LTR were chemically synthesized with a loop nucleotide TTTGTGTAG gap between 21 nucleotide sense and antisense strands having the restriction enzyme sites *SalI* and *XbaI* attached at the 5' and 3' ends for cloning, respectively. The target sequence for the Rev open reading frame (ORF) of HIV-1 is from a previously described target site (Li *et al.*, 2003). The two target sequences, L1 and L2, in HIV-1 5' untranslated region (UTR) are ACTGCTTAAGCCTCAATAATA (nt 60-77) and GCUUGCCUUGAGUGCUCAAAG (nt77-95), respectively. Each shRNA was cloned into *SalI* and *XbaI* sites of the pol III promoter expression system pTZU6+1 (pU6) (Paul *et al.*, 2002) and designated as pU6-R, pU6-L1, and pU6-L2. The construction of pU6R1*siv (previously designated as pU6R1) was described previously (Park *et al.*, 2005). Pre-micro RNA for each shRNA was generated by synthesizing shRNA flanked with the 5' and 3' microRNA 30 (miR30) regions to mimic an miRNA structure as well as to be processed as an miRNA as described previously (Zeng and Cullen, 2005; Zhou, Xia, and Xu, 2005). The sequences for the hybrid L1, L2, and R shRNAs designed were 5'

CAGTGAGCGCCTGCTTAAGCCTCAATAATAGTGAAGCCACAGATGTATTATTGA

GGCTTAAGCAGTTGCCTACTG3', 5'
 CAGTGAGCGTCTTGCCTTGAGTGCTCAAAGTAGTGAAGCCACAGATGTACTTTG
 AGCACTCAAGGCAAGCTGCCTACTG3', and 5'
 CAGTGAGCGCGGAGACAGCGACGAAGAGCTAGTAAGCCACAGATGTAGCTC
 TTCGTCGCTGTCTCCGCTTGCCTACTG3', respectively. The HIV LTR regulated Tat
 dependent expression system (HL) was constructed as described previously (Unwalla *et al.*,
 2006; Unwalla *et al.*, 2004). The U3 sequence extended to TAR of HIV-1 LTR was prepared
 by PCR using sense and antisense primers, 5'- ttctcatcgattggaaggctaattggtc-3' and 5'-
 ttctcatcgatattagtgtggaagttgacagc-3', respectively. The sequence including minimal heat shock
 promoter from *Drosophila* and minimal polyA (Unwalla *et al.*, 2006; Unwalla *et al.*, 2004)
 was chemically synthesized and cloned downstream the TAR region and subsequently the GFP
 coding sequence placed at the 3' end of the minimal poly A region creating plasmid HL (Figure
 2). For transcription termination the simian virus 40 (SV40) poly A signal was placed
 downstream GFP. In HLdTar or HLdHsp the TAR region or the minimal heat shock promoter
 is deleted, respectively, from the parental construct HL. Each of the chemically synthesized
 miRNAs were cloned into HL at the restriction sites *Xba*I and *Spe*I between the minimal poly
 A and GFP generating plasmids HL-L1, HL-L2, or HL-R containing the L1, L2, or R (rev)
 shRNA, respectively. In HL-L1R or HL-L2R two hybrid shRNAs, a combination of L1 and R
 or L2 and R, were placed in a single vector. An intervening sequence size of 52 nucleotides
 long was placed between the two hybrid shRNAs. The HIV proviral construct pNL4-3 was
 kindly provided by NIH AIDS Research and Reference Reagent Program. All cloned constructs
 were confirmed by sequencing double-stranded DNA templates using the Perkin-Elmer
 Applied Biosystem Division (PE/ABD) 373 A automated sequencer.

Foamy virus vectors were constructed using the simian foamy virus vector type 1, isolated
 from rhesus macaque designated as SFVmac (formerly known as SFV-1). SFVmac Tat
 inducible expression vectors were constructed by digesting each shRNA expression cassette
 with the restriction enzyme *Cla*I and *Not*I from HL, HLdTar, HL-L1, HL-L2, HL-R, and HL-
 L2R. These cassettes were then blunt ended and cloned into SFVmac back bone vector,
 pEGFPD, generating SFV/HL, SFV/HLdTar, SFV/HL-L1, SFV/HL-L2, SFV/HL-R, and SFV/
 HL-L2R. The plasmid pEGFPD was derived from the SFVmac vector pCCGL. The
 construction of pCCGL was described previously (Park, Nadeau, and Mergia, 2002). The
 translation initiator ATG codons in the gag and pol genes of construct pCCGL were mutated
 to stop codon TAG generating pEGFPD. Additional mutations were also included in the gag
 gene of pEGFPD with sequence changes GAA GCC at positions 686-691 to GAT TAG, ATG
 at positions 914-916 to TAG, and AGA at positions 1551-1553 to TAG. The L2 and R hybrid
 shRNAs were placed under the control of CMV immediate early gene promoter and cloned
 into pEGFPD generating SFV/CMVL2R for constitutive expression of R and L2 miRNAs.
 The plasmid pTATz expressing HIV-1 Tat was constructed by inserting the HIV-1 Tat coding
 sequence downstream of a CMV promoter.

siRNA blot hybridization

Small size RNA molecules were isolated from cells transfected with shRNA or miRNA
 expression plasmids using the MirVana RNA isolation kit (Ambion, Inc., Austin, TX). Thirty
 micrograms of RNA were denatured with 1M glyoxal and 50% DMSO in 10 mM sodium
 phosphate buffer, pH 7.0, at 50°C for an hour. RNAs were analyzed on a 3 % Nusieve (3:1)
 agarose gel in sodium phosphate buffer (pH 7.0). The RNA was transferred to Hybond- N+
 membrane (Amersham Bioscience UK Limited, England) using a Mini Trans-Blot
 electrophoretic transfer cell (BioRad, Hercules, CA) and hybridized to the [γ -³²P]ATP-labeled
 19 nucleotide shRNA specific antisense probe. For the detection of internal control RNA, U6
 RNA specific antisense oligonucleotide, 5'-TGCTAATCTTCTCTGTATCGT-3' was used as
 a probe after labeling the 5'end with [γ -³²P]ATP.

Tat inducible expression

Transfections of Hela cells or Hela cells expressing HIV Tat were carried out in six well plates by the calcium phosphate method. Cells were seeded at a density of 4.5×10^5 in 6 well plates and transfected with 2ug of each construct carrying the miRNA. Tat inducible GFP expression was also examined in 293 T cells by co-transfecting the Tat expression plasmid (pTATz). The medium was replaced with fresh DMEM containing 10% FBS twenty-four hours after transfection. Cells were monitored for GFP expression at 488 nm wave length excitation, 3-4 days post infection under a microscope with ultraviolet source visualized; with the number of fluorescent cells being scored in the presence and absence of Tat. In addition inducible expression of each miRNA was monitored by Northern blot hybridization using specific probe for each miRNA in the presence and absence of Tat as described above.

HIV inhibition assay

One microgram of each plasmid containing the shRNA or miRNA was co-transfected with 0.5ug pNL4-3 into 293T cells using the calcium phosphate method. Virus production was monitored by reverse transcriptase (RT) activity assay. Briefly, 10 micro liters of supernatant was taken after centrifuging virus infected cell culture for 10 minutes at 10,000 rpm to remove cell debris. The supernatant was mixed in 50 ul of 50 mM Tris-HCl (pH 8), NP-40 (0.1%), dithiothreitol (1mM), KCl (40 mM), EGTA (0.5 mM), MgCl₂ (1mM), and BSA (100 ug/ml). RT activity was assayed in triplicate with ³H-dTTP as a precursor and poly rA/oligo dT as a template/primer.

SFVmac vector production, transduction, and HIV challenges

SFVmac vector particles were produced by co-transfecting 293T cells with SFVmac vector, containing anti-HIV sequences, and the packaging plasmids pCIgag, pCIpol, and pCIenv. In the packaging constructs the coding sequence of gag, pol, and env were cloned separately under the control of the CMV promoter into the pCI vector (Invitrogen). The coding sequence gag was cloned at the restriction enzyme sites *EcoRI* and *BamHI*. The coding sequence for pol or env was cloned at the restriction enzyme sites *EcoRI* and *Asp718*. To generate vector particles 20ug of vector was transfected with packaging plasmids pCIgag (20ug), pCIpol (4ug), and pCIenv (2ug) per T75 cell culture flask into 293T cells. Viral supernatants were harvested 4 days post transfection and were first clarified by centrifugation at 5000 rpm for 20 minutes and then by filtering through a 0.45um filter. The vector particles were further concentrated 100-fold by using the 150 kDa Apollo Centrifugal Spin Concentrators (Orbital Biosciences, Topsfield, MA). The amounts of SFVmac vector produced were titered on fresh 293T cells plated at a density of 2.5×10^4 per well in 24 well plates. Seventy-two hours after infection cells were monitored and scored for GFP fluorescence under a microscope with UV light source. Transduction of U87.CD4.CXCR4 cells was performed by spin-inoculation as previously described (Mergia *et al.*, 2001). Briefly, cells were seeded at a density of 5×10^5 per well, centrifuged at room temperature for 10 minutes at 2,500 rpm and resuspended in 300ul medium containing SFVmac vector particles (multiplicity of infection 10). The resuspended cells were then spun at 1,500 rpm for one hour as described previously (Bahner *et al.*, 1996; Bunnell *et al.*, 1995) and the infection procedure was repeated 8 hours later. The U87.CD4.CXCR4 cells transduced with SFVmac vector containing anti-HIV miRNA were challenged with HIV at a multiplicity of infection of 0.005, 48 hours post transduction, using the DEAE-dextran method as reported previously with minor modifications (Vzorov and Compans, 2000). Briefly, cells were treated with 20 ug/ml DEAE-dextran for two hours and washed with RPMI medium before HIV infection. Two hours after infection, the medium was replaced with fresh DMEM medium supplemented with 15% FBS. The HIV infected cells in triplicate wells of a 6-well plate were incubated at 37°C under 5% CO₂ atmosphere and culture supernatants were collected every two days. The level of HIV production from SFVmac vector transduced cells

were monitored for the amount of capsid p24 by the HIV antigen capture assay kit (Zeptomatrix, Buffalo, NY) according to the manufacturer's directions.

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References

- Akkina RK, Walton RM, Chen ML, Li QX, Planelles V, Chen IS. High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *J Virol* 1996;70:2581–2585. [PubMed: 8642689]
- Bahner I, Kearns K, Hao QL, Smogorzewska EM, Kohn DB. Transduction of human CD34+ hematopoietic progenitor cells by a retroviral vector expressing an RRE decoy inhibits human immunodeficiency virus type 1 replication in myelomonocytic cells produced in long-term culture. *J Virol* 1996;70:4352–4360. [PubMed: 8676458]
- Barnor JS, Miyano-Kurosaki N, Yamaguchi K, Abumi Y, Ishikawa K, Yamamoto N. Lentiviral-mediated delivery of combined HIV-1 decoy TAR and Vif siRNA as a single RNA molecule that cleaves to inhibit HIV-1 in transduced cells. *Nucleotides Nucleic Acids* 2005;24:431–434.
- Bauer TRJ, Allen JM, Hai M, Tuschong LM, Khan IF, Olson EM, Adler RL, Burkholder TH, Gu YC, Russell DW, Hickstein DD. Successful treatment of canine leukocyte adhesion deficiency by foamy virus vectors. *Nat Med* 2008;14:93–97. [PubMed: 18157138]
- Björndal A, Deng H, Jansson M, Fiore JR, Colognesi C, Karlsson A, Albert J, Scarlatti G, Littman DR, Fenyo EM. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J Virol* 1997;71:7478–7487. [PubMed: 9311827]
- Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet* 2003 Jul;34(3):263–264. [PubMed: 12796781]
- Bunnell BA, Muul LM, Donahue RE, Blaese RM, Morgan RA. High-efficiency retroviral-mediated gene transfer into human and nonhuman primate peripheral blood lymphocytes. *Proc Natl Acad Sci U S A* 1995;92:7739–43. [PubMed: 7644487]
- Chi JT, Chang HY, Wang NN, Chang DS, Dunphy N, Brown PO. Genomewide view of gene silencing by small interfering RNAs. *Proc Natl Acad Sci U S A* 2003;100:6343–6346. [PubMed: 12730368]
- Dropulic B, Hermankova M, Pitha P. A conditionally replicating HIV-1 vector interferes with wild-type HIV-1 replication and spread. *Proc Natl Acad Sci USA* 1996;93:11103–11108. [PubMed: 8855316]
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;411:494–498. [PubMed: 11373684]
- Hamilton AJ, Baulcombe DC. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 1999;286:950–952.
- Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 2000;404:293–296. [PubMed: 10749213]
- Hannon GJ. RNA interference. *Nature* 2002;418:244–251. [PubMed: 12110901]
- Hendrie PC, Huo Y, Stolitenko RB, Russell DW. A rapid and quantitative assay for measuring neighboring gene activation by vector proviruses. *Mol Ther* 2008;16:534–540. [PubMed: 18209733]
- Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS. Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* 2003;21:635–637. [PubMed: 12754523]
- Jackson AL, Linsley PS. Noise amidst the silence: off-target effects of siRNAs? *TRENDS in Genetics* 2004;20:521–524. [PubMed: 15475108]
- Josephson NC, Vassilopoulos G, Trobridge GD, Priestley GV, Wood BL, Papayannopoulou T, Russell DW. Transduction of human NOD/SCID-repopulating cells with both lymphoid and myeloid potential by foamy virus vectors. *Proc Natl Acad Sci U S A* 2002;99:8295–8300. [PubMed: 12060773]

- Kiem HP, Allen J, Trobridge G, Olson E, Keyser K, Peterson L, Russell DW. Foamy-virus-mediated gene transfer to canine repopulating cells. *Blood* 2007;109:65–70. [PubMed: 16968897]
- Klase Z, Kale O, Winograd R, Gupta MV, Heydarian M, Berro R, McCaffrey T, Kashanchi F. HIV-1 TAR element is processed by Dicer to yield a viral micro-RNA involved in chromatin remodeling of the viral LTR. *BMC Mol Biol* 2007;8:63. [PubMed: 17663774]
- Lee MT, Coburn GA, McClure MO, Cullen BR. Inhibition of human immunodeficiency virus type 1 replication in primary macrophages by using Tat- or CCR5-specific small interfering RNAs expressed from a lentivirus vector. *J Virol* 2003;77:11964–11977. [PubMed: 14581533]
- Lee NS, Dohjima T, Bauer G, Li H, Li MJ, Ehsani A, Salvaterra P, Rossi J. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol* 2002;20:446–448. [PubMed: 11981553]
- Li MJ, Bauer G, Michienzi A, Yee JK, Lee NS, Kim J, Li S, Castanotto D, Zaia J, Rossi JJ. Inhibition of HIV-1 infection by lentiviral vectors expressing Pol III-promoted anti-HIV RNAs. *Mol Ther* 2003;8:196–206. [PubMed: 12907142]
- Lin X, Ruan X, Anderson MG, McDowell JA, Kroeger PE, Fesik SW, Shen Y. siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. *Nucleic Acids Res* 2005;33:4527–4535. [PubMed: 16091630]
- Liszewicz J, Zeng G, Gratas C, Weinstein JN, Lori F. Combination gene therapy: synergistic inhibition of human immunodeficiency virus Tat and Rev functions by a single RNA molecule. *Hum Gene Ther* 2000;11:807–815. [PubMed: 10779158]
- Malphettes L, Fussenegger M. Macrolide- and tetracycline-adjustable siRNA-mediated gene silencing in mammalian cells using polymerase II-dependent promoter derivatives. *Biotechnol Bioeng* 2004;88:417–425. [PubMed: 15382105]
- Mergia A, Soumya C, Kolson DL, Goodenow MM, Ciccarone T. The efficiency of simian foamy virus vector type-1 (SFV-1) in non-dividing cells and in human PBLs. *Virology* 2001;280:243–252. [PubMed: 11162838]
- Naldini L. Lentiviruses as gene transfer agents for delivery to non-dividing cells. *Curr Opin Biotechnol* 1998;9:457–463. [PubMed: 9821272]
- Novina CD, Murray MF, Dykxhoorn DM, Beresford PJ, Riess J, Lee SK, Collman RG, Lieberman J, Shankar P, Sharp PA. siRNA-directed inhibition of HIV-1 infection. *Nat Med* 2002;8:681–686. [PubMed: 12042777]
- Ouellet DL, Plante I, Landry P, Barat C, Janelle ME, Flamand L, Tremblay MJ, Provost P. Identification of functional microRNAs released through asymmetrical processing of HIV-1 TAR element. *Nucleic Acids Res* 2008;36:2353–2365. [PubMed: 18299284]
- Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* 2002;6:948–958. [PubMed: 11959843]
- Paddison PJ, Caudy AA, Hannon GJ. Stable suppression of gene expression by RNAi in mammalian cells. *Proc Natl Acad Sci U S A* 2002;99:1443–1448. [PubMed: 11818553]
- Paik SY, Banerjee A, Chen CJ, Ye Z, Harmison GG, Schubert M. Defective HIV-1 provirus encoding a multi-target ribozyme inhibits accumulation of spliced and unspliced HIV-1 mRNAs, reduces infectivity of viral progeny, and protects the cells from pathogenesis. *Hum Gene Ther* 1997;8:1115–1123. [PubMed: 9189769]
- Park J, Nadeau P, Zucali JR, Johnson CM, Mergia A. Inhibition of simian immunodeficiency virus by foamy virus vectors expressing siRNAs. *Virology* 2005;343:275–282. [PubMed: 16181654]
- Park J, Nadeau PE, Mergia A. A Minimal Genome Simian Foamy Virus Type 1 (SFV-1) Vector System with Efficient Gene Transfer. *Virology* 2002;302:336–344.
- Parrish S, Fleenor J, Xu S, Mello C, Fire A. Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. *Mol Cell* 2000;6:1077–1087. [PubMed: 11106747]
- Persengiev SP, Zhu X, Green MR. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *RNA* 2004;10:12–18. [PubMed: 14681580]

- Saxena S, Jonsson ZO, Dutta A. Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. *J Biol Chem* 2003;278:44312–44319. [PubMed: 12952966]
- Scacheri PC, Rozenblatt-Rosen O, Caplen NJ, Wolfsberg TG, Umayam L, Lee JC, Hughes CM, Shanmugam KS, Bhattacharjee A, Meyerson M, Collins FS. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc Natl Acad Sci U S A* 2004;101:1892–1897. [PubMed: 14769924]
- Semizarov D, Frost L, Sarthy A, Kroeger P, Halbert DN, Fesik SW. Specificity of short interfering RNA determined through gene expression signatures. *Proc Natl Acad Sci U S A* 2003;100:6347–6352. [PubMed: 12746500]
- Sharp PA. RNA interference. *Genes Dev* 2001;15:485–490. [PubMed: 11238371]
- Singh SK. RNA interference and its therapeutic potential against HIV infection. *Expert Opin Biol Ther* 2008;8:449–461. [PubMed: 18352849]
- Sledz CA, Holko M, d Veer MJ, Silverman RH, Williams BR. Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* 2003;5:834–839. [PubMed: 12942087]
- Strayer DS, Branco F, Landre J, BouHamdan M, Shaheen F, Pomerantz RJ. Combination genetic therapy to inhibit HIV-1. *Mol Ther* 2002;5:33–41. [PubMed: 11786043]
- Taylor JA, Vojtech L, Bahner I, Kohn DB, Laer DV, Russell DW, Richard RE. Foamy virus vectors expressing anti-HIV transgenes efficiently block HIV-1 replication. *Mol Ther* 2008;16:46–51. [PubMed: 17955023]
- Triboulet R, Mari B, Lin YL, Chable-Bessia C, Bennasser Y, Lebrigand K, Cardinaud B, Maurin T, Barbry P, Baillat V, Reynes J, Corbeau P, Jeang KT, Benkirane M. Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science* 2007 Mar 16;315(5818):1579–1582. [PubMed: 17322031] Epub 2007 Feb 22
- Trobridge GD, Miller DG, Jacobs MA, Allen JM, Kiem HP, Kaul R, Russell DW. Foamy virus vector integration sites in normal human cells. *Proc Natl Acad Sci U S A* 2006;103:1498–1503. [PubMed: 16428288]
- Unwalla HJ, Li HT, Bahner I, Li MJ, Kohn D, Rossi JJ. Novel Pol II Fusion Promoter Directs Human Immunodeficiency Virus Type 1-Inducible Coexpression of a Short Hairpin RNA and Protein. *J Virol* 2006;80:1863–1873. [PubMed: 16439542]
- Unwalla HJ, Li MJ, Kim JD, Li HT, Ehsani A, Alluin J, Rossi JJ. Negative feedback inhibition of HIV-1 by TAT-inducible expression of siRNA. *Nat Biotechnol* 2004;22:1573–1578. [PubMed: 15568018]
- Vzorov AN, Compans RW. Effect of the cytoplasmic domain of the simian immunodeficiency virus envelope protein on incorporation of heterologous envelope proteins and sensitivity to neutralization. *J Virol* 2000;74:8219–8225. [PubMed: 10954518]
- Weinberg MS, Morris KV. Are viral-encoded microRNAs mediating latent HIV-1 infection? *DNA Cell Biol* 2006;25:223–231. [PubMed: 16629595]
- Wiznerowicz M, Trono D. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. *J Virol* 2003;77:8957–8961. [PubMed: 12885912]
- Yang D, Lu H, Erickson JW. Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. *Curr Biol* 2000;10:1191–2000. [PubMed: 11050387]
- Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 2000;101:25–33. [PubMed: 10778853]
- Zeng Y, Cullen BR. Efficient processing of primary microRNA hairpins by Drosha requires flanking nonstructured RNA sequences. *J Biol Chem* 2005;280:27595–25603. [PubMed: 15932881]
- Zhou H, Xia XG, Xu Z. An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. *Nucleic Acids Res* 2005;33:e62. [PubMed: 15805121]
- Zucali JR, Ciccarone T, Kelley V, Park J, Johnson CM, Mergia A. Transduction of Umbilical Cord Blood CD 34+ NOD/SCID Repopulating Cells by Simian Foamy Virus Type 1 (SFV-1) Vector. *Virology* 2002;302:229–335. [PubMed: 12441067]

FIGURE 1. Inhibition of HIV replication by shRNAs that target the 5' UTR and rev gene
 (A) Schematic representation of shRNA expression cassette. Each shRNA was expressed under the control of PolIII U6 snRNA promoter. The sense and antisense strand of the siRNA were separated by nine nucleotides and five T nucleotides were attached at the 3' end as a termination sequence. (B) Northern blot analysis of Pol III U6 promoter driven L1, L2, or rev shRNA that target the 5' UTR region and the rev gene. RNA was isolated from 293T cells two days post-transfection. Ten micrograms of small size RNAs were eluted on 12 % urea polyacrylamide gel and transferred to Hybond-N+ membrane (Amersham). The top panel is blot hybridized with a [γ - 32 P]ATP-labeled antisense strand of L1, L2, or R siRNA. The bottom panel in each gel transfer was probed with U6 RNA-specific oligonucleotide probe for RNA recovery. Lane 1 represents RNA samples from cells transfected with plasmid containing the U6 promoter only. The RNA sample from cells receiving the L1, L2, or R expression cassette is in Lane 2 of each blot. The numbers shown are nucleotide (nt) size markers. The sizes of processed shRNA were determined with RNA size markers. (C) Inhibition of HIV replication by shRNA. Each of the shRNA expression cassettes that target the 5' UTR (L1 or L2) or R were co-transfected with pNL4-3 HIV provirus DNA and inhibition of virus replication was monitored by reverse transcriptase assay.

FIGURE 2. Conditional expression of hybrid shRNAs that target HIV

(A). Schematic representation of HIV promoter Tat dependent expression of GFP and shRNAs. The expression vector is constructed as described previously (Unwalla *et al.*, 2006; Unwalla *et al.*, 2004). The top panel represents the LTR region of HIV and bottom panel shows promoter-TAR region of the vector derived from the LTR and the minimal Hsp promoter-minimal polyA region. Each of the shRNAs was placed between minimal Hsp promoter-minimal polyA region and GFP. (B). Tat inducible expression of GFP. Cells were transfected with each construct in the presence and absence of Tat and GFP expression was monitored 48 hours later. HL is the backbone vector for cloning inducible miRNA expression. HLdTar or HLdHsp represents HL with a TAR or Hsp deletion. pSP72 is an unrelated negative control plasmid. (C). Northern blot analysis of Tat inducible shRNA expression. Each blot was hybridized with a [γ - 32 P]ATP-labeled antisense strand of siRNA corresponding to L1, L2, or R. HL-L1, HL-L2, and HL-R are expression cassettes containing shRNAs corresponding to L1, L2, and R. In HL-L2R two shRNAs (L2 and R) were placed consecutively, with 52 nucleotides long intervening sequence between the two shRNAs, in HL vector. The numbers shown are nucleotide (nt) size markers. RNA recovery was monitored by hybridization with U6 RNA-specific oligonucleotide probe.

FIGURE 3. Inhibition of HIV replication by TAR and inducible shRNAs

Each Tat dependant shRNA expression construct was transfected into 293T cells with pNL4-3 HIV provirus and monitored for inhibition of HIV replication over a 4 day period by reverse transcriptase assay. HL-L1, HL-L2, and HL-R are expression cassettes containing hybrid shRNAs corresponding to L1, L2, and R. In HL-L1R two shRNAs (L1 and R) were placed contiguously in HL vector. Controls are constructs without shRNA (HL), TAR deleted without shRNA (HLdTar), or Hsp deleted without shRNA (HLdHsp). Additional controls are mock or only pNL4-3 transfected cells.

FIGURE 4. Inducible expression of shRNAs that target HIV in cells transduced with foamy virus vectors

(A). Shown is a schematic representation of SFVmac vector containing a Tat inducible shRNA expression cassette that targets HIV. (B). Tat regulated GFP expression in 293T cells transduced with foamy virus vectors. 293T cells were transduced with SFVmac vector particles containing shRNAs that target HIV with GFP expression being monitored in the presence or absence of Tat. SFV/HL-L1, SFV/HL-L2, and SFV/HL-R are SFVmac vectors containing a Tat dependent shRNA of L1, L2, and R. SFV/HL is an SFVmac vector containing the Tat dependent expression system without shRNA. (C). Tat regulated shRNA expression in 293T cells transduced with foamy virus vector. Cells were transduced with foamy virus vectors and subsequently transfected with Tat expressing plasmid. Small size RNAs were isolated, blotted, and hybridized with specific probe corresponding to L1, L2, or R. SFV/HL-L1, SFV/HL-L2, and SFV/HL-R are SFVmac vectors containing Tat dependent expression of shRNA of L1, L2, and R. SFV/HL-L2R contains two consecutively placed shRNAs, with 52 nucleotides long intervening sequence, for Tat regulated expression. SFV/HL is SFVmac vector containing Tat dependent expression system without shRNA. The numbers shown are nucleotide (nt) size markers.

FIGURE 5. Inducible inhibition of HIV replication by foamy virus vector expressing miRNAs and by TAR in the vector that is a component of the induction system

U87.CD4.CXCR4 cells were transduced with SFVmac vectors containing shRNAs that are HIV Tat dependent for expression. SFV/HL-R and SFV/HL-L2R are SFVmac vectors containing Tat dependent expression of L2 and consecutive L2 and R shRNAs, respectively. SFV/CMVL2R represents SFVmac vector containing L2 and R shRNAs under the control of CMV promoter for constitutive expression. These transduced cells were challenged with NL4-3 HIV at an moi of 0.005. Samples were harvested at the indicated time intervals in triplicate and assayed for the HIV encoded p24 by ELISA at the indicated time intervals. The p24 level is shown in pg/ml (10^7).

FIGURE 6. Synergistic effect of TAR and TAR-Tat dependent expressed shRNAs on HIV replication

To demonstrate whether there is a synergistic effect by TAR expressed from the vector and TAR-tat dependent expressed shRNAs the results from cells transduced with TAR alone (SFV/HL) were compared with that of cells receiving shRNAs alone (SFV/CMVL2R) or TAR plus shRNAs (SFV/HL-R and SFV/HL-L2R). Inhibition of HIV with SFV/HL, SFV/CMVL2R, SFV/HL-R, or SFV/HL-L2R was more than 98% when compared to cells that received HIV infection only (Figure 5). Comparisons for inhibition of virus replication between samples was performed by independent sample t-test and pair wise comparisons were calculated using the Mann-Whitney U test. Values were considered statistically significant for analyses where $P < 0.05$. * denotes statistically significant results showing the synergistic effect of TAR and shRNAs compared to TAR or shRNAs alone.