Molecular Therapy
Nucleic Acids
Original Article

Efficacy, accumulation, and transcriptional profile of anti-HIV shRNAs expressed from human U6, 7SK, and H1 promoters

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The expression of short hairpin RNAs (shRNAs) in cells has many potential therapeutic applications, including as a functional cure for HIV. The RNA polymerase III promoters H1, 7SK, and U6 have all been used to express shRNAs. However, there have been no direct and simultaneous comparisons of shRNA potency, expression level, and transcriptional profile between the promoters. We show that the 7SK and U6 promoters result in higher shRNA levels and potency compared to the H1 promoter but that in transduced T lymphocytes, higher expression levels can also lead to growth defects. We present evidence that Dicer cleavage of shRNAs is measured from the first base pair in the shRNA stem, rather than from the 5′ end as previously shown for structurally related microRNAs. As a result, guide-strand identity was unaffected by variations in 5′ transcription start sites among the different promoters, making expression levels the main determinant of shRNA potency. While all promoters generated shRNAs with variable start sites, the U6 promoter was the most accurate in using its intended +1 position. Our results have implications for the development of therapeutic small RNAs for gene therapy and for our understanding of how shRNAs are processed in cells.

INTRODUCTION

Combination antiretroviral therapy (cART) used to treat human immunodeficiency virus type 1 (HIV-1) infection involves a cocktail of several drugs targeting HIV-1 reverse transcriptase (RT), protease, integrase, and cellular entry receptors.[1] However, cART cannot clear an infection and is associated with multiple short-term and long-term side effects.[2] Alternative treatments for HIV-1 infection that do not require chronic and lifelong drug administration are extremely desirable. Anti-HIV-1 RNA molecules, expressed from a gene introduced into patient cells, provide an alternative approach to treat the infection, with the potential to provide long-term control of HIV-1 replication.[3–7] The goal is to confer cellular resistance to the virus, resembling the cases of the Berlin and London patients where a hematopoietic stem cell (HSC) transplant from a donor harboring a homozygous 32 base pair deletion in the CCR5 gene conferred resistance to HIV and cured the infection.[8–10] HSCs are the ultimate target of gene therapy as they would provide long-term inhibition of viral replication by allowing all future differentiated HIV-1 target cells to carry the viral resistance phenotype. This could be accomplished by using lentiviral vectors to transduce HSCs *ex vivo* with antiviral genes and then transplanting the cells back into the patient.[4,11–14] Antiviral small RNAs are among the top candidates for gene therapy. They include short hairpin RNAs (shRNAs), ribozymes, RNA decoys, RNA aptamers, and U1 interference RNAs.[15–20] To ensure that these RNAs are expressed in an efficient and safe manner, it is important that the promoters used to express them in cells are optimized to maximize antiviral effects and minimize toxic effects.

In eukaryotic cells, three different types of RNA polymerase (Pol) promoters exist and are recognized by an equal number of RNA Pol enzymes. The interaction between enzyme and promoter mediates transcription of protein-coding mRNAs, as well as a diverse array of non-coding RNAs such as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and small nuclear RNAs (snRNAs). Specifically, the Pol I promoters drive the transcription of most rRNAs, while the Pol II promoters generate mRNAs from protein-coding genes and some small RNAs such as snRNAs and microRNAs. Finally, the Pol III promoters, which are separated into three types, transcribe exclusively non-coding RNAs.[21] The type 1 Pol III promoter transcribes the 5S rRNA gene while type 2 transcribes tRNA genes.[22] Unlike type 1 and 2 Pol III promoters that contain part of their transcripts within the promoter sequence, the type 3 Pol III promoters utilize a generally specific +1 transcriptional start site just after the end of the promoter sequence.[23] The termination sequence for RNA Pol III is a stretch of several thymidines (Ts) that results in a variable tail of one to six

Received 10 June 2020; accepted 17 December 2020;
https://doi.org/10.1016/j.omtn.2020.12.022.

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uridines (Us) on the 3′ end of the transcript.[24] The type 3 Pol III promoters are most suited for expressing artificially designed small RNAs that require a defined 5′ and 3′ end, because they are the only human promoters that do not result in the addition of longer extraneous RNA sequences from the transcription start and/or termination signals. Commonly used type 3 Pol III promoters include the U6, H1, and 7SK promoters, which express the U6 snRNA, the RNase P RNA, and the 7SK RNA, respectively.

Several studies have investigated the activity of shRNAs when expressed from the different Pol III promoters. However, the results have not been consistent, with three studies showing that the U6 promoter produces more active shRNAs compared to the H1 promoter,[25–27] another study showing the H1 promoter produces more active shRNAs compared to the U6 and 7SK promoters,[28] and another study showing no difference in the activity of four different shRNAs expressed from the H1, U6, or 7SK promoters.[29] Furthermore, although it is expected that the type 3 RNA Pol III promoters would drive the expression of shRNAs from their intended +1 start sites, recent evidence demonstrates that this is not always the case. For example, one study showed that the initiation site of transcription from human and mouse U6 promoters is affected by the surrounding sequence.[30] Further investigations have elaborated on this to identify how the precise nucleotide (nt) sequence around the +1 position affects the transcriptional efficiency of the Pol III promoters, as well as which exact nt position is used as the transcriptional start site.[31] Accurate expression of designed small RNAs is critical for them to perform their function and to limit potential off-target effects from unintended transcripts. In the case of shRNAs, their processing by the RNA interference (RNAi) machinery takes place at specific nt positions. Since it has been shown that the human Dicer endonuclease processes double-stranded RNAs by measuring ~22 nt downstream of the 5′ end, vastly different RNA molecules could arise when transcription is altered by even a single nt position.[32,33] Having defined start sites for the transcription of ribozymes, decoys, aptamers, and clustered regularly interspaced short palindromic repeats (CRISPR) nuclease guide RNAs is also important for these molecules to function properly.[34,35]

We have previously shown that an shRNA targeting a conserved sequence in HIV-1 RNA coding for the Gag polyprotein can inhibit viral replication from diverse HIV-1 strains.[17] This shRNA targets a site that begins at nt position 1,498 of HIV-1 NL4-3 DNA and was called sh1498. So far, the only shRNA-targeting HIV-1 RNA that has entered clinical trials targets the tat/rev coding region,[4,36] and its target site begins at nt position 5,983 of HIV-1 NL4-3 DNA. In our previous study, we denoted this tat/rev shRNA as sh5983 and identified a similar anti-HIV-1 potency when compared to sh1498 using the H1 promoter to drive the expression of both shRNAs.[17] In this study, we have used the same plasmid backbone (psiRNA Green Fluorescent Protein :: Zeocin (GFP::Zeo), InvivoGen) to directly compare the antiviral potency of sh1498 and sh5983 when expressed from the promoters H1, U6, and 7SK. As the design of vectors with the H1 and U6 promoters included eight nt from the 7SK sequence directly upstream of the intended transcription start site, we also generated plasmids with the natural H1 and U6 sequences at these positions. Our results show that the U6 and 7SK shRNA cassettes are more potent compared to the H1 shRNA cassettes and that this correlates with the expression level of the shRNAs. We also show that changing the eight nt upstream of the transcription start site from the 7SK sequence to the natural H1 and U6 sequences does not enhance the cassette's potency and for the H1 promoter leads to a slight decrease in potency. Finally, by RNA sequencing (RNA-seq) analysis, we identify differences in transcriptional profiles of shRNAs expressed from the different promoters, particularly in the transcriptional start site. Usage of the expected +1 transcriptional start site was most accurate from the U6 promoter, while the accuracy in using this site from the 7SK and H1 promoters was variable based on the specific molecule being transcribed.

RESULTS

Anti-HIV-1 shRNAs are more potent when expressed from the 7SK and U6 promoters compared to the H1 promoter

To evaluate whether the promoter used to express anti-HIV-1 RNAs affects the antiviral activity of these molecules, we expressed the sh1498 and sh5983 molecules from the H1, U6, and 7SK promoters. To rule out nonspecific activity of the shRNAs contributing to the inhibition of HIV-1 production, we also evaluated a non-sense shRNA (shNS) that does not target HIV-1 RNA. The ability of the shRNAs to suppress HIV-1 production was measured after co-transfections with HIV-1 molecular clone pNL4-3 and each plasmid construct in parallel with the corresponding empty plasmid, psiRNA-H1GFP::Zeo, psiRNA-7SKGFP::Zeo, or psiRNA-U6GFP::Zeo. Relative HIV-1 production was estimated by measuring the activity of HIV-1 RT in the cell supernatants normalized to RT activity in the supernatants of cells co-transfected with the empty psiRNA vectors (Figure 1). The shRNAs expressed from the H1 promoter had a 50 % inhhibitory concentraion (IC_{50}) between 0.5 and 5 ng (Figure 1A) while the IC_{50} of the shRNAs expressed from both the 7SK and U6 promoter were about 10-fold lower, between 0.05 and 0.5 ng (Figures 1B and 1C). These results indicate that the 7SK and U6 shRNA cassettes are more potent compared to the H1 shRNA cassettes. HIV-1 production was not significantly affected by the presence of shNS when expressed from any promoter (Figures 1A–1C) and there were no major differences in HIV-1 production in cells co-transfected with the different empty plasmids (Figure S1).

Anti-HIV-1 shRNAs expressed from the different Pol III promoters do not affect cell viability in HEK293T cells

To determine whether the differences in effects on HIV-1 production were related to cytotoxicity, we measured cell viability in human embryonic kidney 293T (HEK293T) cells using a water-soluble tetrazolium salt (WST-1) metabolism assay 2 days after transfection of the different shRNA expression vectors. shRNA cassettes were transfected into HEK293T cells at 300 to 40,000 times their IC_{50} levels (0.05–5 ng; Figure 1). WST-1 metabolism was normalized to the metabolism in cells transfected with the empty psiRNA vectors. Cell viability was not impaired at doses of up to 2 μg when the shRNAs were expressed from any of the promoters (Figures 2A–2C), demonstrating that the

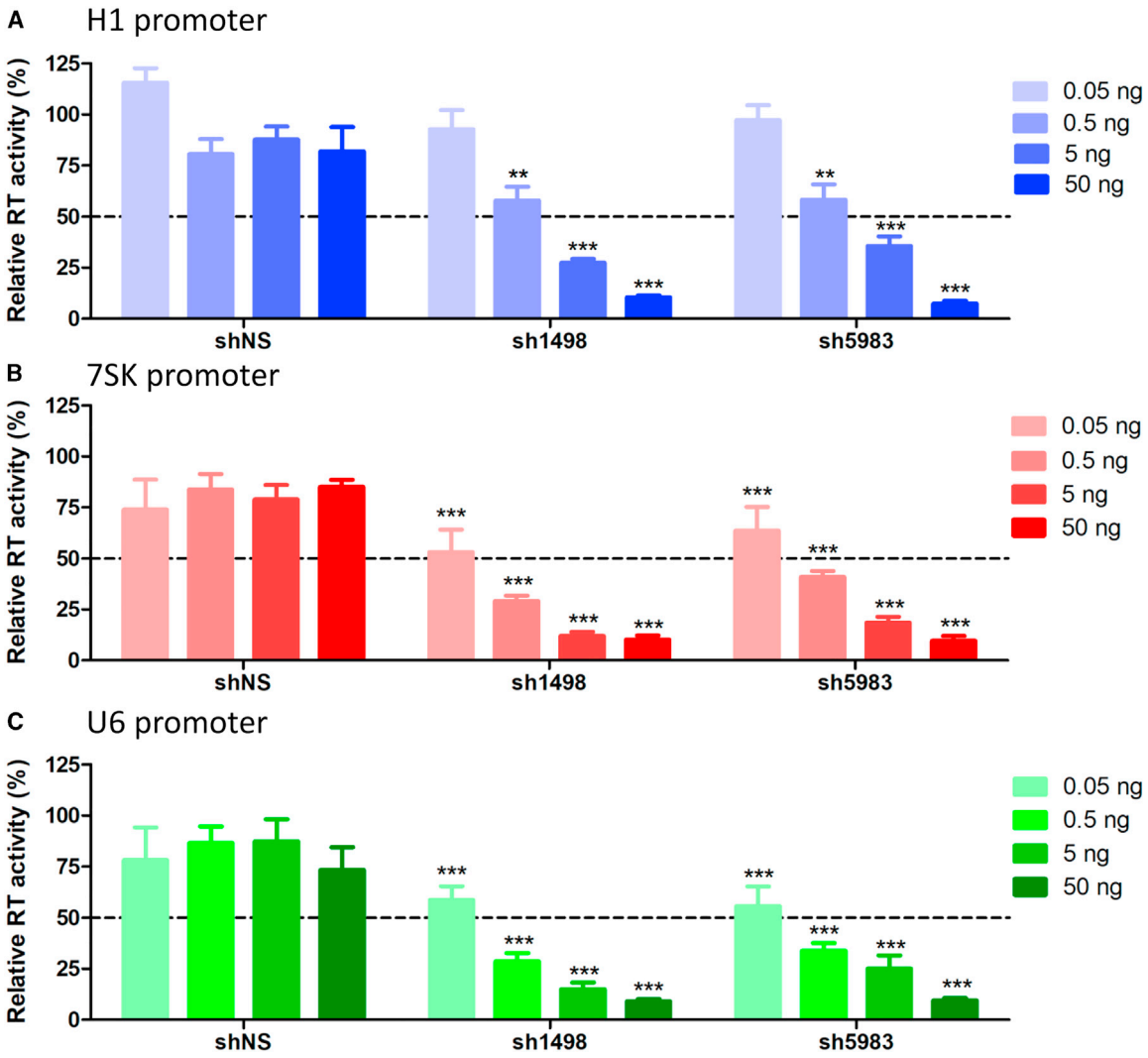

Figure 1. Inhibition of HIV-1 production by anti-HIV shRNAs expressed from different promoters

(A–C) HEK293T cells were cotransfected with the HIV-1 molecular clone pNL4-3 along with one of the plasmids containing the RNA pol III H1 promoter (A), 7SK promoter (B), or U6 promoter (C) and expressing a shRNA. Supernatants were collected 48 h post transfection, and virus production was estimated by measuring HIV-1 RT activity. Data are expressed as a percentage of RT activity in cells cotransfected with the respective empty shRNA expression plasmid. Each data point represents the mean ± standard error of the mean (SEM) from at least two independent experiments with 2 replicates (n = 4–16). The effect of shRNAs on virus production is shown when expressed from each RNA Pol III promoter. A two-way ANOVA with Bonferroni post test was used to compare means to means of empty vector transfected cells. Significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) is shown for those data points that were significantly different.

shRNAs are not cytotoxic when transfected at levels well above their effective amounts in HEK293T cells. These results confirm that differences in the inhibition of HIV-1 production by the shRNAs expressed from the different promoters is not a consequence of differences in cytotoxicity.

The potency of anti-HIV-1 shRNAs is not improved when expressed from Pol III promoters containing the complete natural human sequences

shRNA expression plasmids are typically designed to include a restriction site immediately before the intended +1 transcription start site to facilitate cloning of different shRNA sequences downstream of the promoter (see Table S1 for the upstream sequences in some commonly used vectors). However, the precise start site for transcription can be affected by the surrounding nt sequence[30,31] and these sequences may also affect the transcription efficiency. The commercially available psiRNA plasmid (InvivoGen) used in this study contains the 7SK promoter, which happens to include a KpnI restriction site two nt upstream of the +1 transcription start site. This KpnI site was used by InvivoGen to replace the 7SK promoter with the H1 promoter and by us to replace the 7SK promoter with the U6 promoter for this study. As a result, both the U6 and H1 promoters contain eight nt of

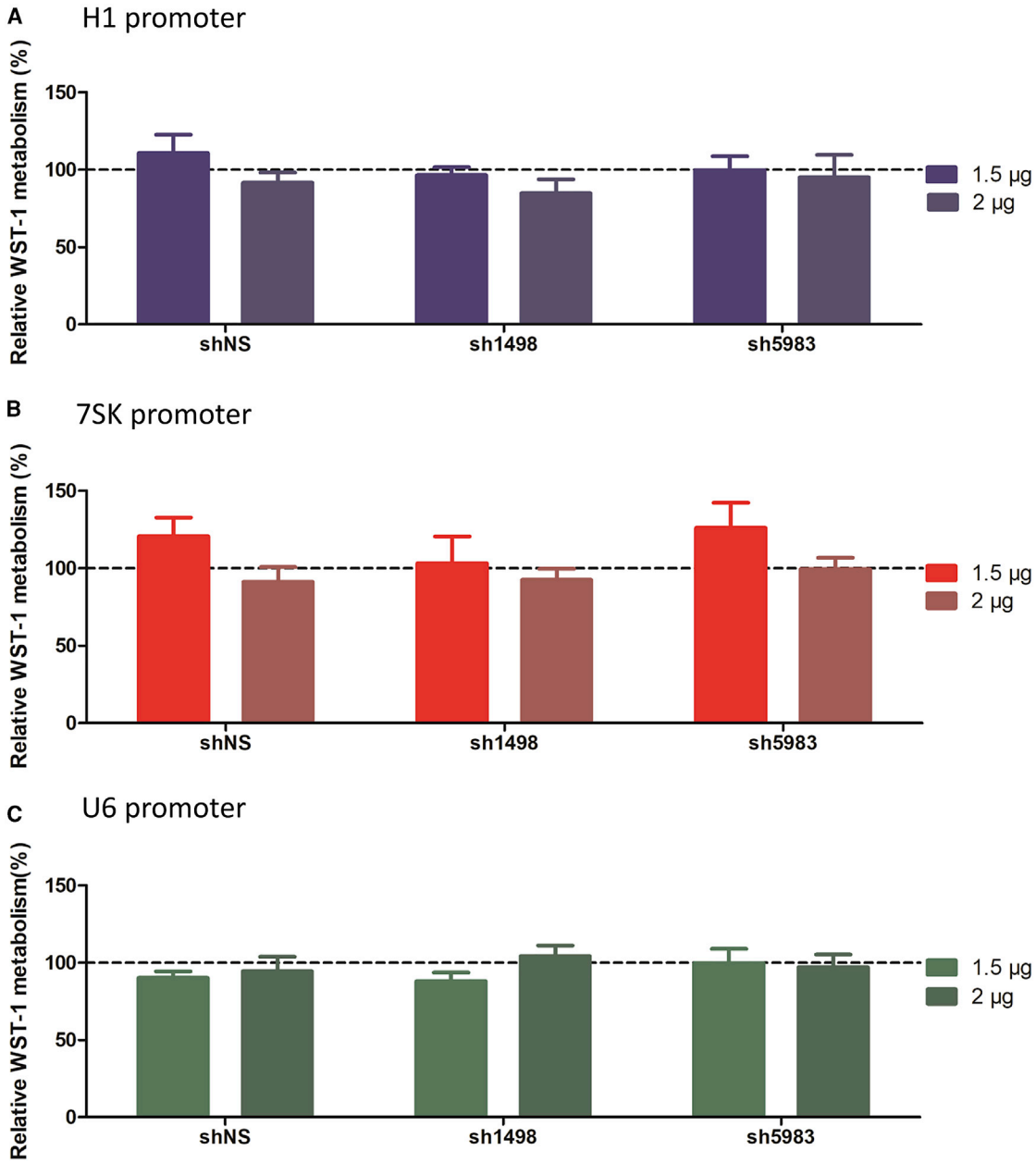

Figure 2. shRNAs do not affect cell viability when expressed from RNA Pol III promoters

(A–C) HEK293T cells were transfected with 1.5–2 µg of plasmid containing the RNA Pol III H1 promoter (A), 7SK promoter (B), and U6 promoter (C) and expressing a shRNA. Cell viability was quantified by measuring the metabolism of WST-1 48 h post transfection. Effect of shRNAs on cell viability is expressed as a percentage of WST-1 metabolism. Each data point represents the mean ± SEM from at least three independent experiments with 1 or 2 replicates (n = 5–8). A two-way ANOVA with Bonferroni post test was used to compare means to means of empty vector transfected cells. No data points were significantly different ($p < 0.05$).

the 7SK promoter sequence directly upstream of their intended +1 transcription start sites (Figure 3A). To evaluate whether the antiviral potency of the shRNAs is affected by these eight nt, site-directed mutagenesis was used to change them to the natural H1 and U6 promoter sequences, which we called the "humanized (h)" hH1 and hU6 promoters (Figure 3A). Inhibition of HIV-1 production was measured using HIV-1 RT activity following co-transfections of plasmid constructs expressing anti-HIV-1 shRNAs and HIV-1 pNL4-3. All data were normalized to the empty psiRNA vectors at each dose. Interestingly, the H1 shRNA cassettes with the 7SK sequence upstream of the transcription start site were more potent compared to the hH1 shRNA cassettes with the natural H1 sequence (Figure 3B). The IC_{50}s of the H1

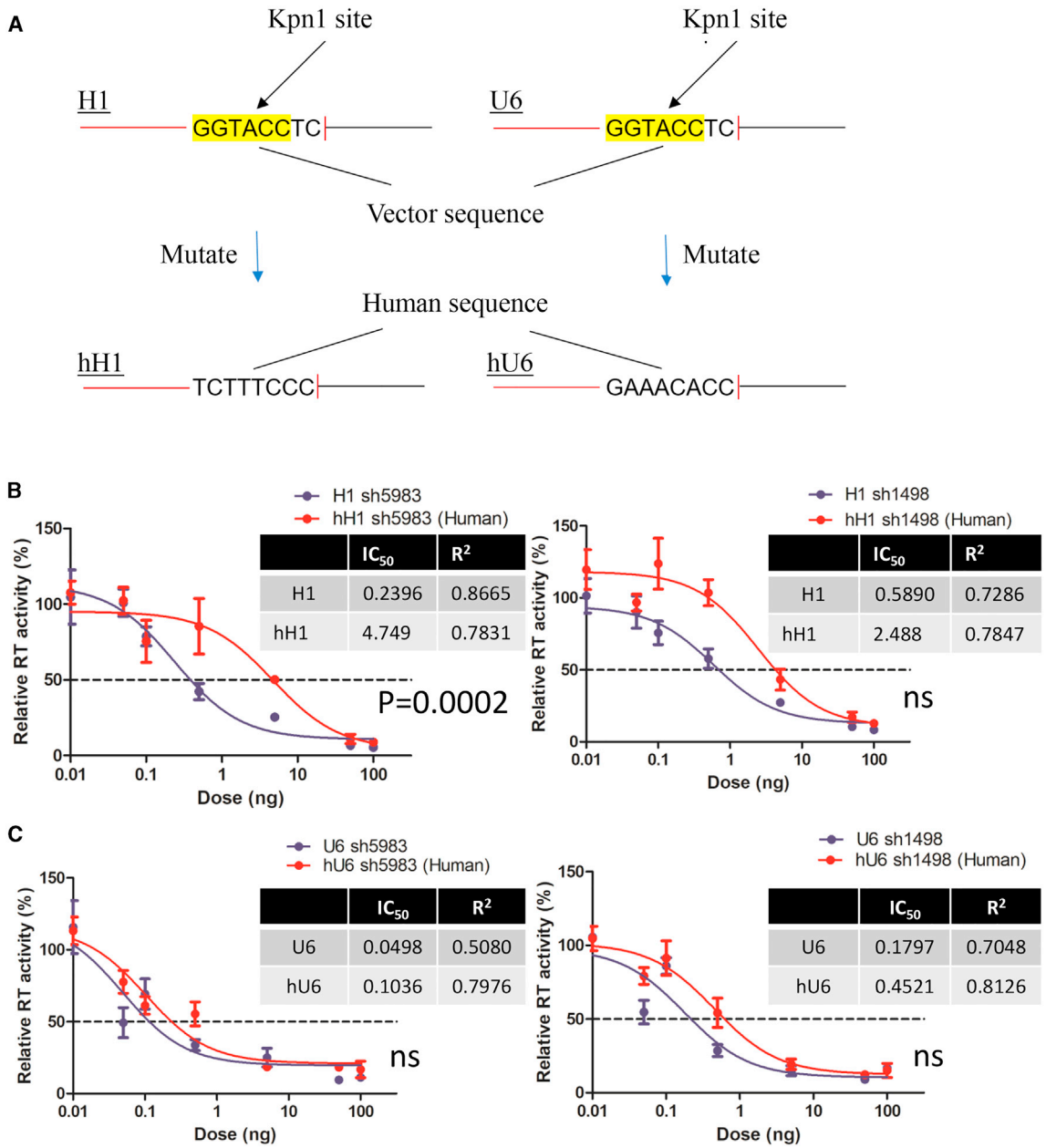

Figure 3. Antiviral effect of shRNAs when expressed from "humanized" promoters

(A) The KpnI sites upstream of the transcriptional start site were replaced by the natural promoter sequence. The effect of the molecules on HIV-1 production was measured exactly as in Figure 1. (B and C) Antiviral potency of shRNAs was compared when expressed from promoters containing the KpnI site and "humanized" promoters for H1 (B) and U6 (C). Each data point represents the mean ± SEM from at least two independent experiments with 1 or 2 replicates (n = 3–15). A nonlinear regression log (inhibitor) versus response equation with least-square (ordinary) fit was applied to the log transformed data, and statistical significance between LogIC_{50}s was determined using extra sum-of-squares F test where $p < 0.05$ is considered not significant (ns).

shRNA cassettes were 0.24 ng for sh5983 and 0.59 ng for sh1498 compared to the IC_{50}s of the hH1 shRNA cassettes, which were 4.75 ng for sh5983 and 2.49 ng for sh1498. In contrast, the IC_{50}s of the U6 shRNA cassettes were similar between the hU6 and U6 promoters (Figure 3C).

Expression levels of shRNAs from each Pol III promoter correlates with their antiviral activity

We then hypothesized that the differences in antiviral potency of the shRNA cassettes with the different Pol III promoters may be due to differing efficiencies of transcription. To determine whether this is

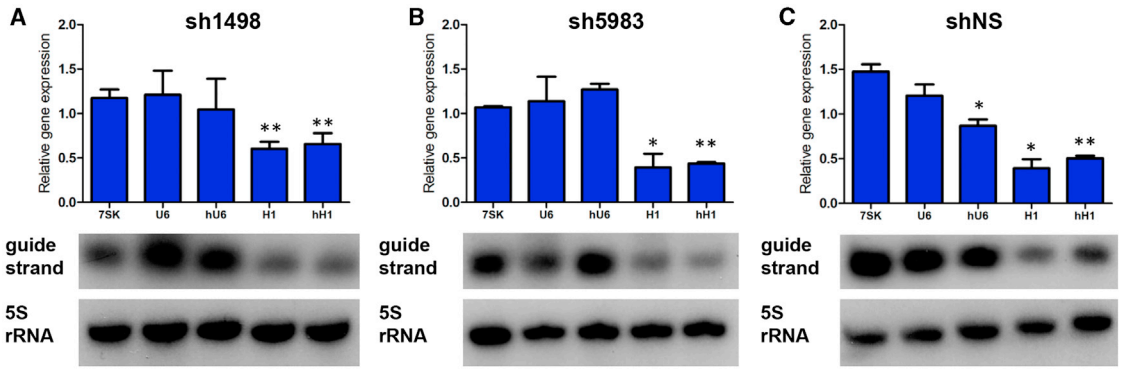

Figure 4. The 7SK, U6, and hU6 promoters express higher levels of shRNA guide strands than the H1 and hH1 promoters

RNA harvested from shRNA-transfected HEK293T cells was migrated in a 15% polyacrylamide-urea gel. shRNA and 5S RNA were detected with ^{32}P-labeled RNA probes and band intensities were analyzed with Fiji software to generate numerical values from two independent experiments. (A–C) Intended guide-strand RNA expression levels from the Pol III promoters is shown for sh1498 (A), sh5983 (B), and shNS (C). Each data point represents the mean ± SEM from two independent experiments (n = 2). An unpaired two-tailed t test was used to compare the mean of 7SK gene-expression values to the mean of the other promoters. Significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) is shown for gene-expression means that were significantly different compared to the 7SK gene-expression means.

the case, we recovered RNA from the transfected cells and measured the expected shRNA guide strands by northern blot. The relative expression level of shNS, sh5983, and sh1498 when expressed by the different Pol III promoters was then determined by quantifying the different band intensities normalized to the 5S rRNA loading control using Fiji software (Figure 4). We observed that gene-expression levels were similar between the 7SK, U6, and hU6 promoters when they express the three different shRNAs. The expression levels between the H1 and the hH1 promoters expressing these same shRNAs were similar but much lower compared to 7SK, U6, and hU6 promoters (Figures 4A–4C). This expression pattern closely mirrors the trend of antiviral potency observed when the shRNAs were expressed from the different Pol III promoters (Figures 1 and 3), which suggests that the differences in antiviral effects observed is most likely due to the varying transcription efficiencies of the promoters.

Cells transduced with a U6- and 7SK-driven anti-HIV-1 shRNA restrict viral replication but have a severe growth disadvantage compared to untransduced cells

To compare the efficacy of an anti-HIV-1 shRNA-driven by the different Pol III promoters against HIV-1 replication in a T lymphocyte cell line, we transduced SupT1 cells with lentiviral vectors (HIV-7-GFP[37]) carrying U6-, 7SK-, and H1-driven sh1498 and shNS. Following transduction, cells were sorted for GFP expression (gating shown in Figure S2A) and infected with HIV-1 NL4-3. Viral replication was measured using HIV-1 RT activity in the culture supernatants over time. Regardless of the promoter, shNS-transduced cells had similar replication kinetics compared to the empty vector transduced cells (Figure 5A). Cells transduced with H1-driven sh1498 also had similar HIV-1 replication kinetics compared to the empty vector (Figure 5B). In contrast, no viral replication was detected in cells transduced with U6- or 7SK-driven sh1498 up to 60 days post infection. Similar results were obtained from an independent transduction (gating shown in Figure S2B) except that one replicate infection for U6 sh1498-transduced cells started to produce detectable RT activity at 26 days post infection (Figure 5C).

To evaluate the potential for shRNA expression to affect cell growth, we also mixed GFP-sorted transduced SupT1 cells with non-transduced SupT1 cells that had also been passed through the flow cytometer (same cells as for Figure 5C but not infected, gating shown in Figure S2B). We then followed the percentage of GFP-positive cells over time using a competitive cell growth assay similar to a previously described method[38] (Figure 5D). Mixed cultures of empty vector, H1 shNS, and 7SK shNS remained at around 50% out to 57 days post mixing. In contrast, the percentage of GFP-positive cells decreased over time for U6 shNS and H1 sh1498 cultures down to 11% and 21%, respectively. They rapidly decreased to nearly 0% in U6 sh1498 and 7SK sh1498 cultures by day 16. Because of the severe impact on cell growth observed for U6 and 7SK sh1498 mixed cultures (Figure 5D), it is difficult to conclude whether the failure of HIV-1 to replicate in these cells (Figures 5B and 5C) was a result of cell toxicity or the effect of the shRNAs. Likely, a combination of shRNAs targeting HIV-1 and loss of cells available to be infected because of toxicity stopped HIV-1 from establishing an infection at the beginning of the experiment.

Expression of shRNAs leads to the accumulation of multiple RNA sequences

shRNA expression cassettes are typically designed with the intended passenger strand at the 5′ end separated by a loop from the intended guide strand at the 3′ end. Following transcription, the complementary passenger and guide strands hybridize to form a hairpin structure, which is exported to the cytoplasm by Exportin 5 where the molecule will be processed by RNAi enzymes. This processing begins by Dicer removing the loop and then by Ago2 cleaving the passenger strand.[16] The guide strand can then direct Ago2 to cleave its target RNA by complementary base pairing. The accumulation of different

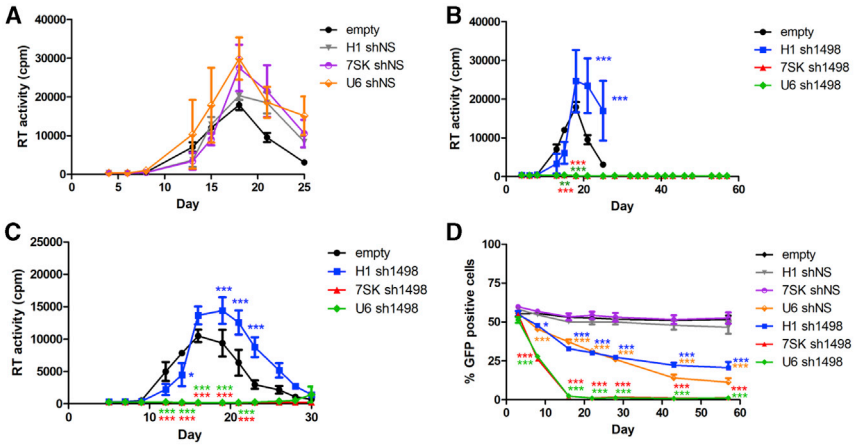

Figure 5. HIV-1 replication is restricted in SupT1 cells expressing sh1498 from the 7SK and U6 promoters, but the cells have a severe growth disadvantage compared to untransduced SupT1 cells

(A–C) SupT1 cells were transduced with HIV-7-EGFP lentiviral vectors expressing shNS (A) or sh1498 (B and C) from the H1, U6, and 7SK promoters and infected with HIV-1 NL4-3 at 1,750 cpm/mL. The mean RT activity (cpm) was measured in culture supernatants at various days post infection. Each data point represents the mean ± SEM from three infections (n = 3). (D) Transduced SupT1 cells were mixed with untransduced SupT1 cells and the percentage of GFP-positive cells was measured at various days post mixing. Each data point represents the mean ± SEM from three experiments (n = 3). A two-way ANOVA with Bonferroni post test was used to compare means to means of empty vector transduced cells. Significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) is shown for those data points that were significantly different from the empty vector transduced controls.

RNA species, in addition to the intended active guide strand, results from the differential processing by the RNAi enzymes, as well as from the potential for alternative start and end sites of transcription. To identify potential differences in the expression of these RNA sequences between the different Pol III promoters, we sequenced the small RNAs of shRNA-transfected HEK293T cells.

RNA-seq data were obtained from cDNA libraries generated with a small RNA library preparation kit that uses adaptors that specifically ligate to 3′ hydroxyl groups that are a hallmark of Dicer cleavage. All sequences that aligned with each shRNA cassette were identified and organized by read number. The data were then expressed as a percentage of reads over total number of reads for each sequence identified. Using a cut-off of 0.1% of total reads, the sequences were organized into tables with their corresponding abundance tabulated for each promoter (Tables S2–S4). The most common sequences (>0.5% of total reads) are illustrated for sh1498 and sh5983 in Tables 1 and 2, respectively. As expected, several sequences were identified for shRNAs expressed from all promoters corresponding predominantly to guide and passenger strands of different lengths. Notably, a more diverse set of sequences were observed for sh5983 compared to sh1498, due predominantly to more diverse Dicer cleavage sites seen at the 3′ ends of passenger strands and the 5′ ends of guide strands. Overall, the results demonstrate that a variety of sequences accumulate following shRNA transfection and that Dicer cleavage is more uniform for sh1498 compared to sh5983.

The U6 promoter is the most accurate in using the +1 transcriptional start site but guide-strand identity is not affected by differences in the +1 site usage

Using the abundance values in Tables S2–S4 we calculated the percentage of reads corresponding to passenger, guide, and other strands, where other strands were defined as those strands that start after the first 3 nt following the expected +1 transcription start site (+4 and over) and end before the first nt preceding the transcription termination signal of five Ts (Figure 6A). For all shRNAs expressed from all promoters, guide strands were the most abundant sequences. For both sh1498 and sh5983, the proportion of guide-strand reads ranged from about 60% to 80%, whereas for shNS almost all the reads corresponded to guide strands and this was consistent for all of the promoters.

The percentage of reads with different end sites and start sites was calculated as a fraction of the total number of the guide strands and passenger strands, respectively (Figures 6B and 6C). For all shRNAs expressed from all promoters, the most common end sequence was UUU, suggesting that regardless of the promoter or shRNA sequences, UUU is the most common shRNA tail. No major differences in transcription termination were evident between the promoters nor the different shRNA sequences. In contrast, the different promoters resulted in major differences in transcription start sites (Figure 6C). Both versions of the U6 promoter were extremely precise at generating transcripts of all shRNAs from the intended +1 transcriptional start site. Meanwhile, the 7SK and H1 promoters varied in their use of this site depending on which shRNA was transcribed. Specifically, the 7SK promoter was reliable in using the +1 site solely when expressing sh5983, whereas the H1 promoter was reliable in using this site when expressing sh5983 and shNS, but not sh1498. The hH1 promoter was reliable in using the +1 site only when transcribing sh1498. Overall, the U6 and hU6 promoters gave rise to more accurate transcriptional start sites when expressing the different shRNAs.

An unexpected observation was that despite differences in transcriptional start sites between the promoters (Figure 6C) there were not major differences in the identity of the guide strands (Tables 1 and 2), suggesting that Dicer cleavage is unaffected by the length of the shRNA 5′ end. This observation can most easily be appreciated when looking at sh1498 sequences (Table 1) for which, despite major differences in the start site between the different promoters, there are only two main Dicer cleavage sites revealed when looking at the 5′ end

Table 1. Transcriptional profile of sh1498 expressed from different RNA Pol III promoters

sh1498	Passenger / Loop / Guide	Promoter (% of total reads)				
	NNNGCAGGAACTACTAGTACCCT**ACTCGAGA**AGGGTACTAGTAGTTCCTGC**TTTTT**	7SK	U6	hU6	H1	hH1
Passenger	CTCGCAGGAACTACTAGTACCCT	1.3				
	CTCGCAGGAACTACTAGTACCC	0.9				
	TCGCAGGAACTACTAGTACCCT	11.6			6.7	
	TCGCAGGAACTACTAGTACCC	1.7			1.2	
	CGCAGGAACTACTAGTACCCT	1.8	0.8	2.8	4.6	2.1
	CGCAGGAACTACTAGTACCC				0.8	
	GCAGGAACTACTAGTACCCTA		0.5			
	GCAGGAACTACTAGTACCCT	7.3	11.2	11.1	7.2	7.9
	GCAGGAACTACTAGTACCC	1.9	3.6	3.7	2.8	2.2
	GCAGGAACTACTAGTACC		0.5			
	GCAGGAACTACTAGTA		0.7		0.7	
	GCAGGAACTACTAGT	0.5	1.6	0.9	1.3	0.6
	GCAGGAACTACTAG		1.4	0.8	1.5	
	GCAGGAACTACT		2.4	1.3	2.6	0.5
Other	AGAAGGGTACTAGTAG		1.4	0.5	0.8	
	AGAAGGGTACTAGTAGT		1.2		0.7	
Guide	GGTACTAGTAGTTCCTGCT	3.2	8.5	8.2	9.1	4.8
	GTACTAGTAGTTCCTGCT		2.0	1.6	2.0	0.6
	GGTACTAGTAGTTCCTGCTT	7.9	11.7	11.7	7.7	10.3
	GTACTAGTAGTTCCTGCTT	0.5	1.5	1.3	1.2	0.7
	GGTACTAGTAGTTCCTGCTTT	33.8	22.6	26.8	18.8	38.6
	GTACTAGTAGTTCCTGCTTT	4.1	6.3	6.3	5.9	5.4
	GGTACTAGTAGTTCCTGCTTTT	7.0	4.2	5.8	5.3	8.4
	GTACTAGTAGTTCCTGCTTTT	8.8	7.4	9.1	11.4	11.4
	GGTACTAGTAGTTCCTGCTTTTT	0.9	0.5	0.7	0.5	1.0
	GTACTAGTAGTTCCTGCTTTTT	1.4	0.9	1.1	0.9	1.7

of the guide strands. Similarly, when looking at the 3′ end of the passenger strands, two major Dicer cleavage sites are apparent for passenger strands that start at the −3 to +1 positions. These results suggest that, for shRNAs, Dicer cleavage sites are measured from the first base pair of the duplex rather than from the 5′ end as has previously been demonstrated for structurally related microRNAs.[32]

DISCUSSION

The RNA Pol III type 3 promoters U6, H1, and 7SK are typically used to express small RNAs such as shRNAs and CRISPR guide RNAs because transcription from these promoters has defined start and end sites and results in the addition of only a few uridines to the 3′ end of transcripts. However, there has been limited work comparing these promoters for the expression of therapeutic RNAs. Additionally, of the studies that have utilized Pol III promoters for gene therapy, most have only focused on one or two promoters at a time.[25,39,40] For gene therapy to treat HIV-1 infection, multiple antiviral RNAs will be needed to avoid the development of viral resistance.[15,18,28,29,41,42] If a single promoter is used to express a combination of antiviral RNAs, deletions of the therapeutic genes could occur as a consequence of recombination between the different transcriptional units using the same Pol III promoter.[28,29] Therefore, a different Pol III promoter expressing each therapeutic RNA may be required to avoid recombination. With this in mind, it is important to properly evaluate which promoter expresses the most active antiviral RNAs, as well as to characterize the transcriptional profile of the promoters U6, 7SK, and H1.

Reports on which Pol III type 3 promoter expresses the most active shRNAs have not been consistent. Specifically, an shRNA targeting the HIV-1 vif coding sequence and a long hairpin RNA targeting the tat/rev viral sequences were shown to be more active when expressed from the H1 promoter compared to both the U6 and 7SK promoters.[28] In another study, two CCR5-specific shRNAs were shown to be more active when expressed from the U6 promoter compared to the H1 promoter, but the U6-driven shRNAs were cytotoxic in primary human blood cells.[25] Similar results were obtained in two other studies where both the expression level and activity of shRNAs were higher for the U6 promoter compared to the H1 promoter.[26,27] In contrast, in a study that compared four different anti-HIV-1 shRNAs expressed from the H1, U6, or 7SK promoters, similar activities were observed regardless of promoter choice.[29] To further complicate comparisons of the different Pol III promoters, it has been found that transcription start sites vary depending on both the promoter choice and the nt upstream of the intended +1 transcription start sites,[30] which can be problematic as these upstream nt are often altered to accommodate a restriction enzyme recognition site (Table S1). In another study comparing several 7SK, H1, and U6 promoters, the nt identity of the +1 transcriptional start site of a small unstructured RNA was found to affect both transcriptional activity and start site

Table 2. Transcriptional profile of sh5983 expressed from different RNA Pol III promoters

sh5983	Passenger / Loop / Guide	Promoter (% of total reads)				
	NNNGCGGAGACAGCGACGAAGAG**GCTCGAGG**CTCTTCGTCGCTGTCTCCGC**TTTTT**	7SK	U6	hU6	H1	hH1
Passenger	CCCGCGGAGACAGCGACGAAGA					4.6
	CTCGCGGAGACAGCGACGAAGA	1.0				
	CCCGCGGAGACAGCGACGAAG					1.1
	CCGCGGAGACAGCGACGAAGAG					1.6
	TCGCGGAGACAGCGACGAAGAG	0.6				
	CCGCGGAGACAGCGACGAAGA					1.8
	TCGCGGAGACAGCGACGAAGA	0.8				
	CCGCGGAGACAGCGACGAAG					0.7
	CGCGGAGACAGCGACGAAGAGG				0.9	
	CGCGGAGACAGCGACGAAGAG				0.9	0.7
	CGCGGAGACAGCGACGAAGA			0.6	0.9	1.0
	GCGGAGACAGCGACGAAGAGGCTCG		0.8	0.5	1.4	
	GCGGAGACAGCGACGAAGAGGCT				0.5	
	GCGGAGACAGCGACGAAGAGGC	1.5	2.7	2.5	5.4	1.1
	GCGGAGACAGCGACGAAGAGG		0.6	0.7	1.3	
	GCGGAGACAGCGACGAAGAG	0.7	0.7	0.9	1.2	
	GCGGAGACAGCGACGAAGA	5.5	6.5	9.7	9.9	3.6
	GCGGAGACAGCGACGAAG	2.6	3.9	6.1	6.7	1.9
	GCGGAGACAGCGACGAA		0.9	0.9	0.8	
	GCGGAGACAGCGACGA		0.6	0.8	0.8	
	GCGGAGACAGCG		2.3	1.1	1.0	
Other	AGACAGCGACGAAGAGGC	0.1	2.8	1.2	2.8	
	ACGAAGAGGCTCG		0.5			
	TCGAGGCTCTTCGTCGC		1.3	0.7	1.2	
	CGAGGCTCTTCGTCGC		0.8		0.7	
	CGAGGCTCTTCGTCGCT		0.6			
	AGGCTCTTCGTCGCTGTCTCC		0.7			
	AGGCTCTTCGTCGCTGTCTCCG		0.6		0.5	
Guide	AGGCTCTTCGTCGCTGTCTCCGCT		0.9		0.7	
	CTCTTCGTCGCTGTCTCCGCT	0.8	1.4	1.1	1.1	
	TCTTCGTCGCTGTCTCCGCT	1.8	4.8	3.3	2.7	1.4
	CTTCGTCGCTGTCTCCGCT	0.8	1.1	0.9	0.8	0.7
	AGGCTCTTCGTCGCTGTCTCCGCTT		1.6	0.9	2.5	
	CTCTTCGTCGCTGTCTCCGCTT	1.8	1.6	1.3	1.3	1.0
	TCTTCGTCGCTGTCTCCGCTT	6.9	9.5	8.3	7.3	6.6
	CTTCGTCGCTGTCTCCGCTT	5.4	4.6	4.7	3.7	5.2
	CTCTTCGTCGCTGTCTCCGCTTT	1.2	0.7	0.7	0.6	0.7
	TCTTCGTCGCTGTCTCCGCTTT	16.6	9.5	10.8	8.5	15.0
	CTTCGTCGCTGTCTCCGCTTT	26.7	13.7	16.9	12.2	26.3
	TTCGTCGCTGTCTCCGCTTT	1.5	1.7	1.9	1.4	1.7
	TCTTCGTCGCTGTCTCCGCTTTT	1.5	1.1	1.5	1.0	1.4
	CTTCGTCGCTGTCTCCGCTTTT	11.6	4.2	7.3	4.8	10.7
	TTCGTCGCTGTCTCCGCTTTT	1.5	0.9	1.3	0.9	1.5
	CTTCGTCGCTGTCTCCGCTTTTT	0.8		0.5		0.8

identity with the H1 promoters resulting in the most variable +1 start site.[31] While important observations have been made, there has not been any direct simultaneous comparison of shRNA activity, expression levels, and transcriptional profile between the U6, 7SK, and H1 promoters.

In this study, the antiviral potencies of an anti-HIV-1 shRNA targeting tat/rev (sh5983) and another targeting Gag (sh1498) were compared when expressed from the 7SK promoter, as well as the U6 and H1 promoters, which included eight nt of the 7SK sequence upstream of the +1 start site (Figure 1) and when expressed from the humanized hU6 and hH1 promoters with the complete natural promoter sequences (Figure 3). Consistent with studies that simultaneously compared both the expression level and activity of H1- and U6-driven shRNAs,[25–27] our results suggest that shRNAs expressed from the U6 promoter are more potent and are expressed at a higher level when compared to shRNAs expressed from the H1 promoter (Figures 1 and 4), regardless of the nt identity

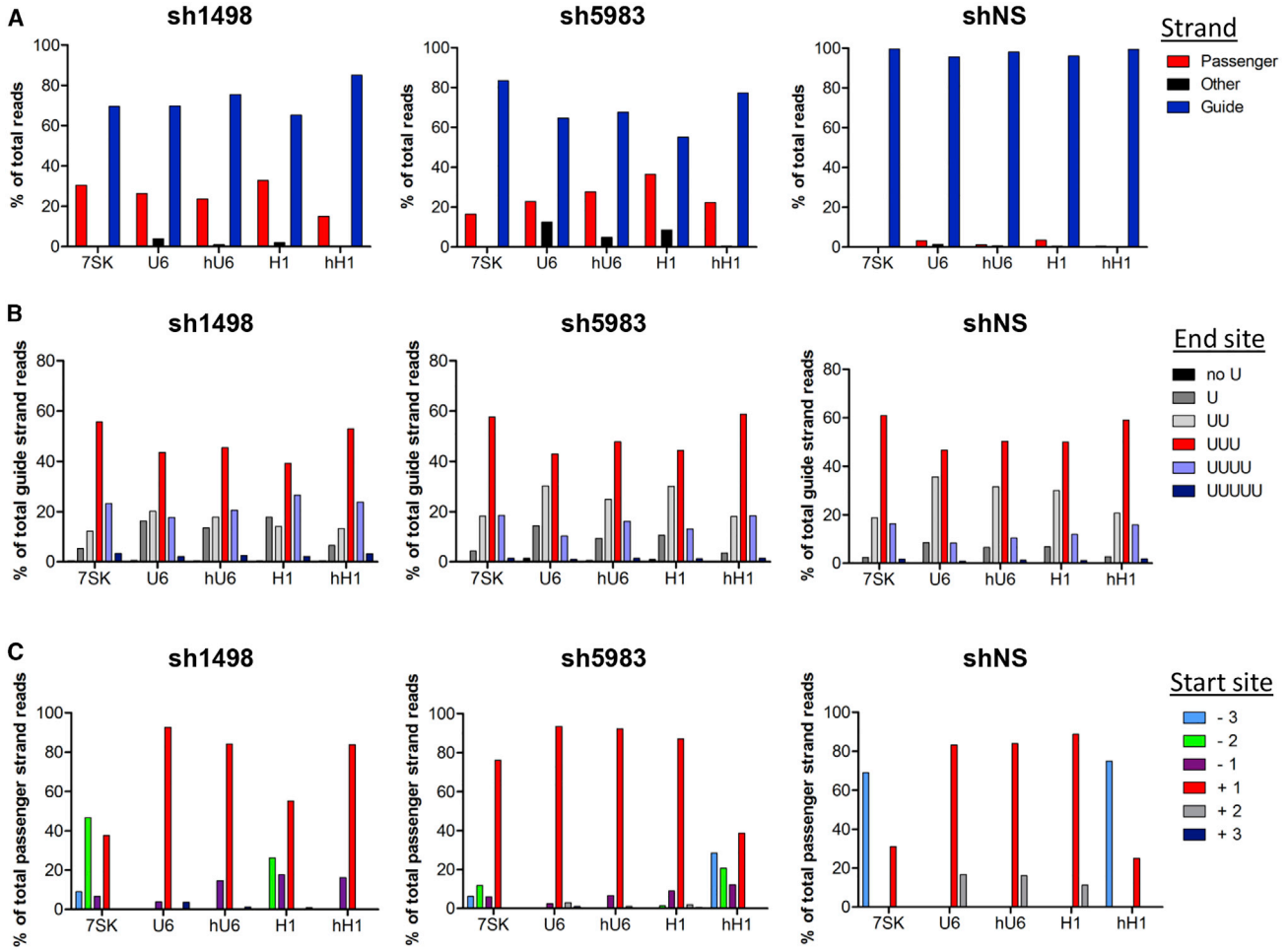

Figure 6. Proportion of reads corresponding to different RNA variants of sh1498, sh5983, and shNS

(A) The proportion of variants corresponding to expected passenger, guide, and other strands is shown. (B) The proportion of guide strands ending in different numbers of Us is shown. (C) The proportion of passenger strands starting at different positions relative to the expected +1 transcriptional start site is shown.

upstream of the transcription start site (Figure 3). While two other studies reported conflicting observations for shRNA activity,[28,29] neither study compared expression levels and the activity level of the shRNAs were compared at only one dose. It is possible that the doses selected in those studies were already above the level required for maximum target suppression and so the differences in activity related to differences in shRNA expression were not apparent. Within our inhibitory assays (Figures 1 and 3) we tested several doses and although the two anti-HIV shRNAs expressed from all promoters were able to almost completely suppress HIV-1 production at the higher doses in these assays, there was a clear and consistent difference in potency, with the H1 promoter shRNA constructs being less potent compared to the U6 and 7SK shRNA constructs. Our data therefore strongly suggest that the U6 promoter is indeed more transcriptionally active compared to the H1 promoter, at least in HEK293T cells. We also show that the 7SK promoter is more transcriptionally active compared to the H1 promoter, providing similar expression levels and activities of shRNAs as the U6 promoter.

Although the transcriptional profiling revealed major differences in the localization of the transcription start sites for the different promoter-shRNA constructs (Figure 6C), our data suggest that the variations in RNA expression levels between the promoters (Figure 4) is the primary contributing factor toward the differences in antiviral potency observed (Figures 1 and 3). Differences in the transcriptional start sites were observed; for example, the 7SK promoter was only accurate in its usage of the +1 site when expressing sh5983 and was much less accurate when expressing sh1498, while the U6 promoter was overall the most accurate in using the +1 transcriptional start site (Figure 6C). Because sh1498 expressed from the 7SK promoter had antiviral capabilities similar to those expressed from the U6 promoter (Figures 1B and 1C), despite its transcription from the 7SK promoter not beginning reliably at the +1 site, we conclude that

expression from the +1 transcription start site is not a primary contributing factor to the antiviral potency. Nonetheless, the identification of U6 as the most accurate promoter will have strong implications for molecules where an accurate +1 transcription start site is required for function, such as for CRISPR guide RNAs. As we compared only three shRNAs in one cell type, additional studies are needed to confirm whether this observation can be widely applied to different molecules and in different cells.

It has been shown that human Dicer measures approximately 22 nt from the 5′ end of microRNAs (5′ counting rule) to locate its cleavage site[32] and would therefore be expected to yield different small interfering RNA (siRNA) duplexes when the +1 site at the 5′ end is changed. The different duplexes should then give rise to different guide strands, which, in contrast with our conclusion, would be expected to have different RNAi activities. However, our sequencing results suggest that Dicer cleavage sites are not altered when the +1 site is changed and that, regardless of the +1 start site, the identity of the guide strands remains the same as can be seen most clearly for sh1498 in Table 1. Since the 5′ counting rule for human Dicer is dependent on a 5′ terminal phosphate, it may be that shRNAs transcribed from RNA Pol III promoters do not follow this rule because they have a 5′ terminal triphosphate instead of the monophosphate typical of primary microRNAs.[32] Alternatively, since the 5′ counting rule was established using *in vitro* cleavage experiments, it may not apply to Dicer cleavage in live cells and further studies are needed to better characterize Dicer cleavage of different hairpin RNAs in different environments. Interestingly, our results also demonstrate that independently from the promoter, Dicer cleavage was more uniform for sh1498 compared to sh5983, with only two major Dicer cleavage sites apparent for sh1498 compared to four for sh5983 (Tables 1 and 2). Thus, the identity of the shRNA sequence can affect the number of Dicer products and, consequently, the diversity of guide and passenger strands. Importantly, our results show that certain promoter-shRNA cassettes have different start sites but that this does not affect the identity and corresponding RNAi activity of the guide strand. Rather, RNAi activity is dependent only on the expression level, with both the U6 and 7SK promoters producing more active shRNAs compared to the H1 promoters.

Although anti-HIV shRNAs are potent inhibitors of viral replication and are among the top candidates for combination anti-HIV gene therapy, there is a need to ensure that they do not negatively impact the cells they are expressed in (HSCs and their progeny cells). A potential negative attribute of using a promoter with a more promiscuous start site is that potential off targeting could be increased due to the increased diversity of the transcripts. While our results suggest that the guide-strand identities are not affected by the more promiscuous transcriptional start sites of some promoter-shRNA combinations, there is certainly an increase in the diversity of the passenger strands, which could be a source of off targeting. Several potential mechanisms of shRNA toxicity have been described, including activation of innate immune responses,[43–45] off-target effects on human RNAs,[46,47] and saturation of components of the RNAi pathway.[48] Two studies showed that adverse effects of shRNAs expressed from the U6 promoter could be prevented by using the H1 promoter instead.[25,26] For this reason, an shRNA-targeting CCR5 expressed from the H1 promoter[40,42] was chosen for a combination anti-HIV gene therapy clinical trial.[49] While toxicity of shRNAs expressed from the U6 promoter has been observed, an shRNA targeting tat/rev expressed from the U6 promoter was shown to be safe in preclinical and clinical studies.[4,36] This suggests that toxicity of U6-promoted shRNAs may be related only to particular shRNA sequences.

We evaluated whether there was cellular toxicity in response to the shRNAs expressed from the different promoters to determine whether the inhibition of HIV-1 production seen in response to co-transfection was a consequence of cell death. Our experiments confirmed that the expression of the different shRNAs does not cause cytotoxicity in HEK293T cells (Figure 2). In contrast, when we transduced different shNS- and sh1498-promoted constructs into SupT1 cells, cytotoxicity was evident for some constructs by a marked decrease in transduced cells (GFP positive) versus untransduced cells (GFP negative) over time (Figure 5D). Regardless of the promoter used, sh1498-transduced cells had a greater growth disadvantage compared to shNS-transduced cells, suggesting that the mechanism of the growth defect in sh1498-transduced cells was at least partially sequence dependent. Interestingly, while the decline in GFP-positive cells was similar for U6 and 7SK sh1498 cultures, only U6 shNS cultures had a noticeable decline with both H1 and 7SK shNS cultures remaining stable at approximately 50% out to 57 days. Further studies will be needed to determine whether the mechanism of the growth defect in GFP-positive cells is a direct toxicity triggered by the different promoter-shRNA combinations and to evaluate whether different results can be obtained using different anti-HIV-1 shRNA sequences.

Although RNA Pol III promoters are typically used to express therapeutic small RNAs such as CRISPR guide RNAs and shRNAs, few studies have compared the three commonly used promoters for efficacy, RNA accumulation, and transcriptional profile of their intended therapeutic products. Using three different shRNAs, we show that both the U6 and 7SK promoters consistently express higher amounts of shRNAs compared to the H1 promoter and that this leads to an increase in potency in the case of the two anti-HIV shRNAs. Consistent with other studies, our RNA-seq data show that all RNA Pol III promoters produce shRNA transcripts with different start[30] and end[24] sites. We also show that the U6 promoter consistently uses the +1 transcriptional start site most frequently but that in a T lymphocyte cell line both an anti-HIV-1 and a non-sense shRNA expressed from this promoter confer a negative impact on cell growth. To the best of our knowledge, this is the first study to use RNA-seq data to examine the Dicer cleavage sites of shRNAs. These results suggest that contrary to established rules for Dicer cleavage in mammalian cells,[32] shRNAs are not cleaved at a set distance from their 5′ ends but rather at a set distance from the first base pair in their stem. Consequently, the guide strands produced from different promoters are largely the same, regardless of how accurate the promoter is at starting transcription at the intended +1 position. Furthermore, we

show that the shRNA sequence affects the uniformity of Dicer cleavage.

Overall, our results highlight the unpredictability of shRNA transcription and processing in human cells, as well as underscore the importance of evaluating different promoters for any particular shRNA gene therapy candidate. For an HIV-1 functional cure using shRNAs, several parameters need to be considered to ensure the safe and effective creation of HIV-1-resistant cells. Our results highlight the importance of shRNA promoter choice and demonstrate that expression level is most important for shRNA activity and toxicity. Testing alternative anti-HIV-1 shRNAs using the different promoters described in this study will be required to identify a combination of shRNA and promoter that is effective in safely generating HIV-1-resistant cells in combination with other anti-HIV-1 RNAs.

MATERIALS AND METHODS

Cell culture

HEK293T cells were grown in Dulbecco's modified Eagle's medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS, HyClone), 50 μg/mL streptomycin, and 50 U/mL penicillin (Life Technologies, Carlsbad, CA, USA). SupT1 cells were grown in Roswell Park Memorial Institute Medium 1640 (HyClone), supplemented with 10% heat-inactivated FBS (HyClone), 50 μg/mL streptomycin, and 50 U/mL penicillin (Life Technologies).

Vector construction

The U6 promoter was amplified by PCR using the pSIREN-shuttle vector (Clontech Laboratories, Mountain View, CA, USA) as the template and the following primers:

Forward: 5′-GCGCTATCGATGGAAGAGGCTATTTCCCA-3′

Reverse: 5′-GCGGAGGTACCGTCCTTTCCACAAGATAT-3′.

These amplicons were digested with Acc651 (isoschisomer of KpnI) and ClaI and then ligated into Acc651- and ClaI-digested psiRNA-7SKGFP::Zeo (InvivoGen, San Diego, CA, USA) plasmid to create psiRNA-U6GFP::Zeo. The DNA inserts coding for the shRNAs were generated by annealing complementary oligonucleotides as described previously.[17,50] These DNA inserts were then ligated into BbsI-digested psiRNA-7SKGFP::Zeo, as well as into BbsI-digested psiRNA-U6GFP::Zeo. Plasmids containing the H1 promoter were constructed in a previous study.[17]

Mutagenesis

All constructed plasmids originating from the psiRNA-H1GFP::Zeo (InvivoGen) and the constructed psiRNA-U6GFP::Zeo plasmids were mutated using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. The primers used in the mutagenesis, with the nt which conferred mutations within the promoter highlighted in bold, are shown in Table S5.

Transfections and RT assay

HEK293T cells were plated into a 96-well plate at 5×10^4 cells/well, 24 h prior to transfections. Co-transfections were performed with 50 ng HIV-1 molecular clone (pNL4-3) and each plasmid construct at 0.05 to 100 ng using TransIT-LT1 (Mirus Bio, Madison, WI, USA) according to the manufacturer's protocol. Culture supernatants were collected 48 h after transfection, and viral production was measured by RT assay as previously described.[51,52] Briefly, 5 μL of supernatant was incubated with 50 μL of RT cocktail containing a poly(A) template (Roche, Basel, Switzerland), an oligo(dT) primer (Life Technologies) and [^{32}P] deoxythymidine triphosphate (dTTP; 3,000 Ci/mmol; Perkin Elmer, Waltham, MA, USA) for 2 h at 37°C. The poly dT RT product was then detected by spotting 5 μL of the reaction mixture onto diethylaminoethyl (DEAE) filter mats (Perkin Elmer), washing away unincorporated [^{32}P] dTTP with 2× SSC, and measuring counts per minute (cpm) on a microplate scintillation counter (MicroBeta TriLux; Perkin Elmer). The amount of HIV-1 RT enzyme in the supernatants is proportional to the cpm readout.

WST-1 assay

HEK293T cells were plated into a 96-well plate at 5×10^4 cells/well, 24 h prior to transfections. Transfections were performed with 1.5 or 2 μg of each plasmid construct and TransIT-LT1 (Mirus) according to the manufacturer's instructions. WST-1 assay (Roche Applied Science, Penzberg, Germany) was performed 48 h after transfection according to the manufacturer's protocol. Cell viability was measured 2 h after the addition of the WST-1 reagent as previously described.[53]

RNA extraction for northern blot

HEK293T cells were plated in a 10 mL tissue culture dish at 3.5×10^6 cells/dish, 24 h prior to the transfections with 1 μg of plasmid. Cell lysates were recovered 48 h after transfection using TRIzol reagent (Life Technologies). Total RNA from the recovered cell lysates was obtained by phenol chloroform extraction followed by cleanup with a miRNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Northern blot

Following the RNA extraction, a total of 15 μg of total RNA from each sample was mixed with equal volume of 2× gel loading buffer. RNA was resolved in a 15% polyacrylamide-urea gel as described previously.[54] Briefly, the RNA was transferred to a neutral charged nylon membrane (Hybond-N, Amersham Biosciences, Little Chalfont, UK) with a semi-dry electroblotter (40 min, 4°C, 20 V). Membranes were prehybridized in prehybridization buffer composed of 6× SSC, 2× Denhardt's solution, and 0.1% SDS. Hybridization of the ^{32}P-labeled RNA probes to the membrane was done at 37°C overnight in hybridization buffer of identical composition as the prehybridization buffer. Once probe hybridization was complete, the membrane was washed for 15 min in wash buffer #1 (2× SSC), 15 min in wash buffer #2 (1× SSC), and 15 min in wash buffer #3 (0.1× SSC), all at 37°C. Northern bands were exposed on radiographic film and analyzed with the Fiji software[55] to generate raw numbers

proportional to the exposure intensity of the bands. The band corresponding to each shRNA was standardized to the band corresponding to the 5S RNA loading control of the samples to generate percentage-based values of the shRNA band intensities compared to the 5S band intensities.

Probe labeling

To generate radioactively labeled RNA probes, we performed *in vitro* transcription using the HiScribe T7 kit (NEB) according to the manufacturer's instructions with annealed template DNA and [α-32P] cytidine triphosphate (CTP, 800 Ci/mmol; Perkin Elmer). Following *in vitro* transcription, the reaction mixture was treated with DNase I (New England Biolabs, Ipswich, MA, USA) to eliminate the template DNA. The labeled RNA probes were then purified with ProbeQuant G50 Micro Columns (GE Healthcare, Little Chalfont, UK) to remove unincorporated nt. The probe sequences with the T7 promoter sequence underlined were as follows:

sh1498-antisense: 5′-GGGTACTAGTAGTTCCTGCCTATAGTGAGTCGTATTAATTTC-3′,

sh5983-antisense: 5′-TCTTCGTCGCTGTCTCCGCCTATAGTGAGTCGTATTAATTTC-3′,

shNS-antisense: 5′-TACGAATGACGTGCGGTACCTATAGTGAGTCGTATTAATTTC-3′,

S-rRNA-antisense: 5′-GGGAATACCGGGTGCTGTAGGCTTTCCTATAGTGAGTCGTATTAATTTC-3′ and T7-promoter-sense: 5′-GAAATTAATACGACTCACTATA-3′.

SupT1 T cell transduction, infection, and competitive growth

Promoter shRNA cassettes were subcloned from the psiRNA plasmids into the lentiviral transfer vector HIV-7-EGFP (donated by Dr. J. Rossi)[37] using forward primer 5′-TATGCGGCCGCAGGGATTTTGGTCATGTTCTTAATCGATACTA-3′ and reverse primer 5′-GTAACGCCTGCAGGTTAATTAAGTCTAGAAGCTTTTCCAA-3′ and restriction sites NotI and XbaI. Lentiviral transfer vectors were cotransfected into HEK293T cells with a plasmid expressing vesicular stomatitis virus G protein (from Dr. J. Rossi) and the packaging plasmid psPAX2 (Addgene, number 12260). The supernatant was collected 48 h post transfection, and the lentiviruses were concentrated using Lenti-X (Clontech Laboratories) following the manufacturer's protocol. Lentivirus titers were determined using the percentage of GFP-positive SupT1 cells transduced with a range of dilutions (1 in 4 to 1 in 2,048). In 5 mL cultures, 1.05×10^6 SupT1 cells were transduced with lentiviruses at a MOI of 1 with 8 μg/mL Polybrene (Sigma-Aldrich, St. Louis, MO, USA). Cells were sorted 72–96 h after transduction for GFP expression using the gates shown in Figures S2A and S2B with a FACSAria Fusion cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). Cells were then plated in 96-well round-bottom plates at 2×10^4 cells/well for HIV-1 infection and for competitive growth. Cells plated for HIV-1 infection were infected with HIV-1 NL4-3 (1,750 cpm/mL, determined using the HIV-1 RT assay) 24 h later, and HIV-1 RT activity was determined on various days post infection in the culture supernatant at which times 100 μL of supernatant was collected and replaced with 100–110 μL of fresh media. Cells plated for competitive growth were immediately mixed with 2×10^4 cells/well of untransduced SupT1 cells that had been passed through the flow cytometer without sorting. GFP-positive cell percentage was determined at various days post mixing using a LSRFortessa flow cytometer (BD Biosciences) at which times 100 μL of cells were collected and replaced with 100–110 μL of fresh media.

RNA extraction for RNA-seq

HEK293T cells were plated in a flask at 5×10^6 cells/flask, 24 h prior to the transfection. The cells were then transfected with 5 μg of plasmids expressing sh1498, shNS, and sh5983 expressed from the promoters U6, 7SK, H1, hU6, or hH1. An individual flask was used to transfect the three shRNAs; one flask was used for each promoter. Cell lysates were recovered after 48 h using TRIzol reagent (Life Technologies). Total RNA was isolated using phenol chloroform extraction followed by cleanup with a RNeasy mini kit (QIAGEN) according to the manufacturer's instructions.

RNA-seq

Libraries were prepared from total RNA by Genome Quebec (Montréal, Canada) using a NEB small RNA library kit with size selection. The libraries were run in an Illumina HiSeq4000 SR50 sequencing lane. Data analysis was carried out by the Canadian Center for Computational Genomics (Montréal, Canada). Briefly, adaptor sequences were clipped from the reads, but reads were not trimmed, to avoid introducing false variants. Reads were mapped to the different promoter shRNA sequences. Reads smaller than 6 base pairs and singletons were removed, and the remaining sequences were arranged by read number. Reads were then expressed as a percentage of total reads for each promoter shRNA sequence (Data S1, S2, S3, S4, and S5). A single table was created for each shRNA, reporting the percentage of total reads for each variant that occurred at greater than 0.1% of total reads (Tables S2–S4).

Statistical analysis

A two-way ANOVA with a Bonferroni post test was used to compare replicate means of each test shRNA to replicate means of empty psiRNA-transfected cells in Figures 1 and 2. The same test was used to compare replicate means of infection and competitive growth time-course data to means of empty vector transduced cells (Figure 5). Nonlinear regression extra sum-of-squares F test was used to compare $LogIC_{50}$s in Figure 3. Unpaired Student's t tests were used to compare mean RT activity and relative gene-expression data in Figures S1 and 4, respectively. Graph Pad Prism Version 5.03 was used to perform all statistical analyses (GraphPad, San Diego, CA, USA).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.12.022.

ACKNOWLEDGMENTS

We would like to thank Dr. John Rossi (City of Hope, Duarte, CA, USA) for provision of lentiviral plasmids. We would also like to thank Christian Young (Lady Davis Institute flow cytometry manager) for assistance in cell sorting and François Lefebvre (Canadian Centre for Computational Genomics Bioinformatics manager) for assistance in RNA-seq workflow design and data analysis. This work was supported by grant PJT-148704 awarded to A.G. and R.J.S. from the Canadian Institutes of Health Research (CIHR). R.P.G. is supported by a doctoral fellowship from the Fonds de Recherche du Québec - Santé (FRQS). O.D.C. and C.M.G.M. were supported by a Frederick Banting and Charles Best Canada Graduate Scholarship-Master's (CGS-M) from the CIHR and by a MSc scholarship from the FRQS, successively. S.P.A.-L. was supported by a doctoral fellowship from the Consejo Nacional de Ciencia y Tecnologia (CONACYT, Mexico). R.J.S. was a recipient of the Wares Family post-doctoral award and a post-doctoral fellowship from the Richard and Edith Strauss Canadian Foundation through the McGill University Department of Medicine.

AUTHOR CONTRIBUTIONS

A.G. and R.J.S. conceived the study. R.J.S. and R.P.G. designed the experiments, analyzed the data, and wrote the manuscript. A.G. revised the manuscript. R.P.G. conducted most of the experiments. O.D.C., C.M.G.M., A.D., S.P.A.-L. and M.J.C. assisted in conducting experiments. All authors read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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