Synergistic Inhibition of HIV-1 Regulatory Function Using a Dual Expression shRNA Plasmid

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HIV infection of $CD4^+$ T Helper lymphocytes results in a gradual loss of immune competence, which ultimately leads to development of the acquired immune deficiency syndrome (AIDS). HIV-1 expresses two regulatory proteins, Tat and Rev, which act to upregulate viral transcription and facilitate mRNA transport enhancing the rate of translation. Inhibition of Tat and Rev have been shown to block virus replication. Therefore, Tat and Rev represent critical key points in the virus replication cycle. This study investigated the potential of RNA interference (RNAi) to inhibit these proteins using short hairpin RNAs (shRNAs). For this, plasmids expressing shRNAs targeting either Tat or Rev and a dual shRNA plasmid concurrently targeting both proteins, were evaluated using a Tat- and Rev-dependent luciferase assay. Results showed that although both individual shRNAs significantly reduced luciferase activity, dual shRNA plasmid exhibited a greater reduction in luciferase activity than either individual shRNA, indicating a synergistic effect.

Introduction

The Human Immunodeficiency Virus (HIV-1) primarily infects $CD4^+$ T-lymphocytes which function to control the immune response (Barre-Sinnousi et al., 1983). The outcome of virus replication in these cells leads to their gradual loss resulting in a concomitant decrease in immune competence. As a result, individuals with untreated HIV-1 infection become increasingly susceptible to opportunistic infections, a condition that ultimately progresses to the acquired immune deficiency syndrome (AIDS) (Pantaleo et al. 1993). Current treatments act to reduce virus replication but are not curable. Therefore, work continues along several fronts to inhibit key viral functions and break the viral replication cycle.

The HIV-1 genome expresses two regulatory genes, *Tat* and *Rev*, that act in concert to control virus replication (Haseltine, 1991). The transactivator of transcription (Tat) is a 14 kDa protein composed of 86 amino acids that functions to upregulate viral transcription from the HIV promoter (Dayton et al. 1986). Tat is formed early in the virus replication cycle from completely spliced mRNAs. Tat functions through its interaction with the transactivation response element (TAR), which is a *cis*-acting RNA element transcribed within the first 59 nucleotides of all nascent viral transcripts. The Tat/TAR interaction leads to recruitment of the host positive elongation factor (pTEFb) to the transcription complex which is localized on the HIV promoter. Once recruited, pTEFb phosphorylates the C-terminal domain of RNA Polymerase II resulting in highly efficient transcription from the HIV provirus promoter (Zhu et al. 1997; Liu et al. 2014). Importantly, in the absence of Tat, HIV transcripts are inefficiently formed, and viral replication does not proceed.

The regulator of expression of virion proteins (Rev) is 19 kDa protein composed of 116 amino acids (Pallard and Malim 1998). Like Tat, Rev is generated from completely spliced viral mRNAs that are produced early in the replication cycle. Rev controls the fate of viral transcripts by mediating their export from the nucleus before they can be completely spliced. Rev acts by binding another *cis*-acting RNA element, the Rev response element (RRE), which is present in all viral transcripts (Pollard and Malim, 1998). The transition from completely to partially to unspliced viral transcripts is dependent on the available amount of Rev. Early in the virus replication cycle, Rev levels are low and viral mRNAs are completely spliced before transport from the nucleus. Translation of these fully spliced transcripts results in increasing levels of Tat and Rev, which are localized back to the host cell's nucleus. Increasing Rev levels eventually result in increased rates of export of viral mRNAs before they can be completely spliced. These partially spliced mRNAs give rise to viral structural proteins, enzymes, and accessory proteins, while unspliced viral mRNAs encode the viral glycoproteins and are packaged into virus capsids prior to budding, thereby completing the virus replication cycle. Like Tat, Rev plays a crucial role in virus replication by influencing the overall success of the viral replication cycle.

The regulatory functions of Tat and Rev during HIV-1 replication make them attractive targets for inhibition through RNA interference. RNAi is a cellular process that controls gene expression by inhibiting specific mRNA functions, leading to a reduction in gene expression and subsequent loss of gene function. (Fire et al. 1998). The RNAi pathway is triggered by the presence of intracellular double-stranded RNA (dsRNA) and is initiated when dsRNAs are recognized and cleaved into short siRNAs by Dicer (Agrawal et al., 2003). The siRNAs subsequently associate with Agonaute to form the RNA-induced silencing complex (RISC) (Zhou et al. 2012). RISC utilizes siRNA to bind the target mRNA via complementary base pairing and cleave the target which silences gene expression (Iwakawa et al. 2021). Importantly, RNAi can also be triggered by endogenous expression of small hairpin RNAs (shRNA) that are designed to target a gene of interest (Jai et al. 2022). Short hairpin RNAs are synthetic RNA molecules that, when transcribed, fold back on themselves to form a hairpin structure. When expressed in cells, shRNAs are recognized by Dicer, processed into siRNA, and incorporated into RISC to facilitate the knockdown of gene expression through RNAi mechanisms (Rao et al. 2009).

Although RNAi has been shown to inhibit various HIV functions (DiGiusto et al. 2010; Zhou et al. 2011) several issues remain. Among these include the method of delivery, maintenance of long-term expression, and selection/optimization of target inhibition. In this study the selection and optimization of target inhibition was studied in experiments designed to compare the effect of siRNAs targeted to HIV-1 Tat and Rev as well as the effect of single versus dual shRNA expression. For this, individual shRNA plasmids were created to target either Tat or Rev mRNA. We then sought to determine if simultaneous expression of these two shRNAs might act synergistically to more completely inhibit the activity of these two viral proteins. For these studies, a shRNA dual plasmid was created and its activity compared to that of single shRNA expression. The activity of each shRNA was assessed using a HIV-1 genomic clone that expresses firefly luciferase under the control of both Tat and Rev. These results showed that while individually targeting HIV-1 Tat or Rev significantly reduced HIV gene expression, greater inhibition was observed in experiments involving dual expression of both shRNAs.

Methods

Plasmids

The HIV-1 *Tat* and *Rev* RNA sequences from the HIV-1 genomic clone NL43 (Accession number M19921) were analyzed for the presence of potential 20-mer siRNA sites using the Oligoengine RNAi design online tool (www.oligoengine.com).One site in *Tat* at nucleotides 5834-5854 and one in *Rev* at nucleotides 8403-8423 were selected for analysis. Each siRNA was used to design a short hairpin RNA (shRNA) that consisted of the 20-nucleotide siRNA (sense) sequence linked to its complementary (antisense) sequence via a short linker sequence. The completed shRNA was converted to a double-stranded DNA (dsDNA)

and BglII and HindIII sites were added to the 5' and 3' ends, respectively. The upper and lower oligonucleotides are shown (Table 1). Each oligonucleotide was synthesized (Eurofins) and annealed to form double-stranded shDNAs which was cloned into the BglII and HindIII sites of pH1.Stuffer (Jureka et al. 2011). The resulting plasmids, pH1.Tat5834 and pH1.Revsh8403 express cloned shRNAs from the RNA Polymerase III H1 promoter. Correct cloning of each plasmid was verified by sequencing (Integrated DNA Technologies). A dual shRNA expression plasmid, pDUOsh5834.8403, was designed to express both Tatsh5834 and Revsh8403. This plasmid expresses Tatsh5834 and Revsh8403 from individual RNA Polymerase III U6 promoters that are oriented in opposite directions to ensure autonomous expression. This plasmid also expresses enhanced Green Fluorescent Protein (eGFP) and hygromycin B kinase from the human phosphoglycerokinase (hPGK) promoter. The plasmid sequence was designed and synthesized by VectorBuilder (https://en.vectorbuilder.com).

pNL43.Luc.R$^-$.E$^-$ (He J et al. 1995; Connor RI et al. 1995), obtained from BEI Resources, is a HIV-1 NL43 reporter plasmid that expresses firefly luciferase in a Tat and Rev dependent manner. pPCR-Script, a derivative of pBluescript, was used as a negative control.

Cells and transfection

RNAi was assessed in transiently transfected HEK293T cells using a HIV-based reported plasmid. HEK293T cells were maintained in Complete Medium (Dulbecco's modification of Eagle's medium containing 10% fetal bovine serum/1% penicillin/streptomycin/1% L-glutamine) and cultured in a humidified incubator at 5% CO_2 and 37°C. For transfection, $1x10^5$ HEK293T cells were seeded in 6-well plates containing 2 mL Complete Medium. The following day, the medium was replaced, and the cells transfected. The effect of shRNA expression was tested using three different shRNA treatments: Tatsh5834, Revsh8403, and DUOsh5834.8403. A negative control of mock transfected cells (no plasmid DNA) and a pNL43.Luc.R$^-$.E$^-$ only positive control were included in each experiment. Transfection mixtures containing 1000 ng pH1.Tatsh5834, pH1.Revsh8403, DUOsh5834.8403 or pPCR-Script (negative control) and 100 ng pNL43.Luc.R$^-$.E$^-$ were combined and adjusted to 100 µL total e with serum-free DMEM before adding 4 µL ViaFect Transfectiovolumn Reagent (Promega). Mock transfected (no plasmid DNA) cells were used to measure any endogenous luminescence from the cells. Transfection mixtures were incubated for 15 minutes at room temperature before being added to the cells. The transfected cells were incubated at 37°C for 48 hours after which the cultures were analyzed for siRNA-mediated inhibition. Each experiment was carried out in triplicate with two biological replicates and three technical replicates for each treatment.

Analysis of siRNA activity

Inhibition of Tat and Rev function was assessed by luciferase assay using a Pierce Luciferase Glow Assay Kit (ThermoFisher Scientific). Briefly, 48 hours post transfection, the medium was removed from each well and the cells were washed with 400 µL phosphate-buffered saline (PBS). The PBS was removed, and the cells were lysed using 1X Cell Lysis buffer. The cells were then incubated for 15 minutes at room temperature on a platform rocker to allow for complete lysis. Triplicate 20 µL samples of each lysate were transferred to a white 96-well plate and 50 µL Glow Assay Buffer containing D-Luciferin was added to each sample. The plate was incubated at room temperature for 10 minutes to allow the establishment of a stable bioluminescence signal, which was then measured using a SpectraMax L luminometer (Molecular Devices). The results from each treatment were analyzed by one-way ANOVA to determine differences between treatments.

Results

In this study, selection/optimization of siRNA-mediated inhibition was investigated by targeting two HIV-1 regulatory proteins (Tat and Rev) and comparing their decreased function using single-target and dual-target endogenous expression. For this, two potential siRNA sites were identified within the HIV-1 NL43 genome sequence (Fig 1A and B). A potential siRNA site within each coding region was located at nucleotides 5834-5854 (*Tat*) and nucleotides 8403-8423 (*Rev*). Each sequence was used to design a corresponding shRNA for plasmid-based endogenous expression. Each shRNA was converted to a dsDNA (shDNA) and BglII and HindIII restriction sites were added for cloning. The completed shDNAs (Fig 1C and D) were synthesized and cloned into pH1.Stuffer forming pH1.Tatsh5834 and pH1.Revsh8403. These plasmids express their respective shDNAs from a RNA Polymerase III H1 promoter (Fig. 2) to allow for endogenous shRNA production. To determine if simultaneous expression of both shRNAs produces synergistic effects, a dual expression shRNA plasmid was designed and synthesized. This plasmid, pDUOsh5834.8403 expresses both Tatsh5834 and Revsh8403 from individual RNA Polymerase III U6 promoters, as well as the green fluorescent protein from the human PGK promoter (Fig. 2).

Table 1. shRNA oligonucleotides.

Tatsh5834 upper	5'GATCCCAGCCAGTAGATCCTAGACTA TTCAAGAGATAGTCTAGGATCTACTGGC TTTTTTA 3'
Tatsh5834 lower	5'AGCTTAAAAAAGCCAGTAGATCCTAG ACTATCTCTTGAATAGTCTAGGATCTAC TGGCTGGG 3'
Revsh8403 upper	5'GATCCCCCCGAAGGAATAGAAGAAGA ATTCAAGAGATTCTTCTTCTATTCCTTCG GTTTTTA 3'
Revsh8403 lower	5'AGCTTAAAAACCGAAGGAATAGAAGA AGAATCTCTTGAATTCTTCTTCTATTCCT TCGGGGG 3'

The use of siRNAs to inhibit Tat and Rev functions was determined by testing three RNAi treatments that targeted Tat or Rev alone or simultaneously. These treatments not only allowed a comparison of the efficacy of targeting Tat versus Rev, but also a comparison between single siRNA expression and dual siRNA expression. In these experiments, each shRNA expression plasmid was co-transfected with pNL43.Luc.R$^-$.E$^-$, in which firefly luciferase expression is controlled by both Tat and Rev. To determine the relative silencing activity of each shRNA, HEK293T cells were transiently co-transfected with 100 ng pNL43.Luc.R$^-$.E$^-$ and 1000 ng shRNA plasmid or control. This plasmid ratio was found to be optimal in balancing the catalytic activity of firefly luciferase because relatively small amounts of Tat and Rev can generate high luciferase signals (data not shown).

Forty-eight hours post transfection, eGFP expression in pDUOsh5834.8403 treated cells was visualized to confirm transfection (Fig 3B), then the cells were lysed and analyzed for relative luciferase activity. Single anti-Tat and anti-Rev shRNA treatments resulted in significantly decreased luciferase activity compared to the positive control across three independent experiments (Revsh8403 and Tatsh5834, Fig. 4A). Notably, the dual shRNA expression appeared to exhibit a synergistic effect, leading to a more significant reduction in luciferase activity compared to single shRNA treatments (Dual shRNA, Fig 4A). To compare relative shRNA inhibition, (+) control luciferase expression was set to 100% and each shRNA treatment was calculated to determine the shRNA Affect, which was indicated as a percentage of the (+) control (Fig. 4B). From this analysis, Tatsh5834 and Revsh8403 were shown to generate a shRNA Affect ranging from 69.4-100% to 70.1-96.4% of the (+) control, respectively. In comparison, dual expression of Tatsh5834 and Revsh8403 resulted in a much greater shRNA Affect that ranged from 38.9-45.7% of the (+) control (Fig 4) Overall, these data indicate that simultaneous targeting of Tat and Rev resulted in higher levels of gene expression compared to targeting either gene alone, suggesting a synergistic effect.

A.

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Query  5761  TGTTTATCCATTTCAGAATTGGGTGTCGACATAGCAGAATAGGCGTTACTCGACAGAGGA  5820

Query  5821  GAGCAAGAAATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAG  5880
                          ||||||||||||||||||||
Sbjct  1     -------------AGCCAGTAGATCCTAGACTA--------------------------  20

Query  5881  CCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGT  5940
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B.

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Query  8341  GCAGGGATATTCACCATTATCGTTTCAGACCCACCTCCCAATCCCGAGGGGACCCGACAG  8400

Query  8401  GCCCGAAGGAATAGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTCGATTAGT  8460
                ||||||||||||||||||||
Sbjct  1     --CCGAAGGAATAGAAGAAGAA--------------------------------------  20

Query  8461  GAACGGATCCTTAGCACTTATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCAGCTACCA  8520
```

C. Tat 5834 shRNA

	BglII	Sense (Target)	Hinge	Antisense	HindIII	
5′	GATCTCC	**AGCCAGTAGATCCTAGACTA**	TTCAAGAGA	**TAGTCTAGGATCTACTGGCT**	TTTTTA	3′
3′	AGG	**TCGGTCATCTAGGATCTGAT**	AAGTTCTCT	**ATCAGATCCTAGATGACCGA**	AAAAATTCGA	5′

D. Rev 8403 shRNA

	BglII	Sense (Target)	Hinge	Antisense	HindIII	
5′	GATCTCC	**CCGAAGGAATAGAAGAAGAA**	TTCAAGAGA	**TTCTTCTTCTATTCCTTCGG**	TTTTTA	3′
3′	GGG	**GGCTTCCTTATCTTCTTCTT**	AAGTTCTCT	**AAGAAGAAGATAAGGAAGCC**	AAAAATTCGA	5′

Figure 1. shRNA design. The HIV-1 NL43 (accession number M19921). *Tat* and *Rev* gene sequences were analyzed for potential siRNA binding sites. Two 20-mer siRNA target sites, one in *Tat* at nucleotides 5834-5854 (A) and one in *Rev* at nucleotides 8403-8423 (B) were selected for analysis. A short hairpin shRNA was designed to target each sequence. Each shRNA consisted of the 20-mer target sequence, and its complement (antisense sequence) linked by a six-nucleotide hinge. The shRNA sequence was converted to dsDNA, then BglII and HindIII partial sites were added for cloning. The completed Tat5836shRNA (C) and Rev8403shRNA (D) were synthesized and annealed to form shDNAs.

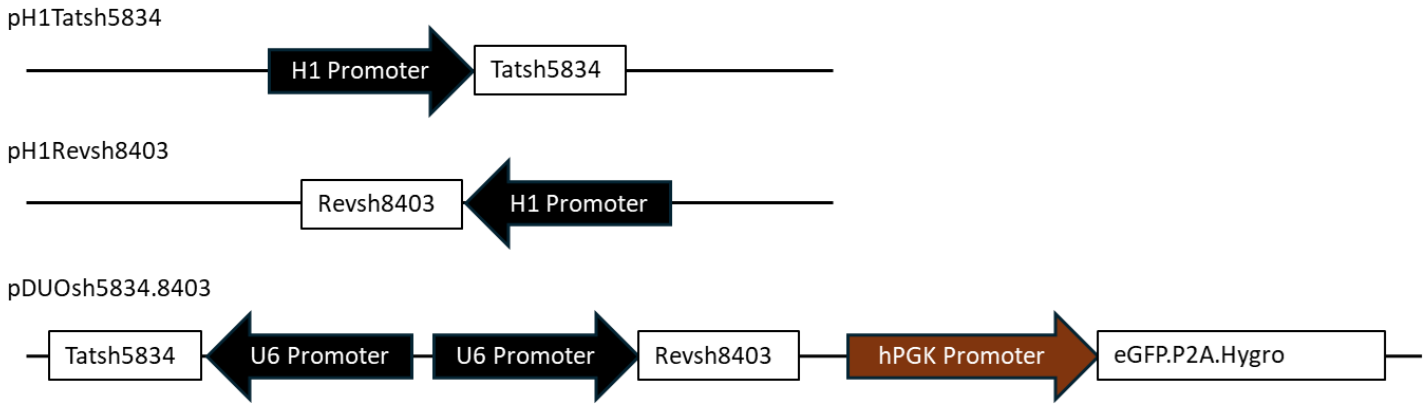

Figure 2. shRNA plasmids. Two shDNAs targeted to HIV-1 Tat and Rev mRNAs were synthesized and cloned into the RNA Polymerase III expression plasmid, pH1.Stuffer. Each single shRNA expression plasmid was generated by inserting a shDNA sequence into pH1.Stuffer which had been linearized using BglII and HindIII. The resulting plasmids, pH1.Tatsh5834 and pH1.Revsh8403 were verified by sequencing. To compare single shRNA expression to simultaneous expression of two shRNAs, a dual shRNA expression plasmid was designed and synthesized. This plasmid, pDUOsh5834.8403 expresses both Tatsh5834 and Revsh8403 from two separate RNA Polymerase III U6 promoters oriented in opposite directions. In addition, pDUOsh5834.8403 expresses both enhanced Green Fluorescent protein and Hygromycin B kinase from the human PGK promoter. Expression of eGFP and Hygromycin B kinase is facilitated by the P2A peptide sequence (Kim et al. 2011) which induces ribosome skipping during translation of the bicistronic mRNA.

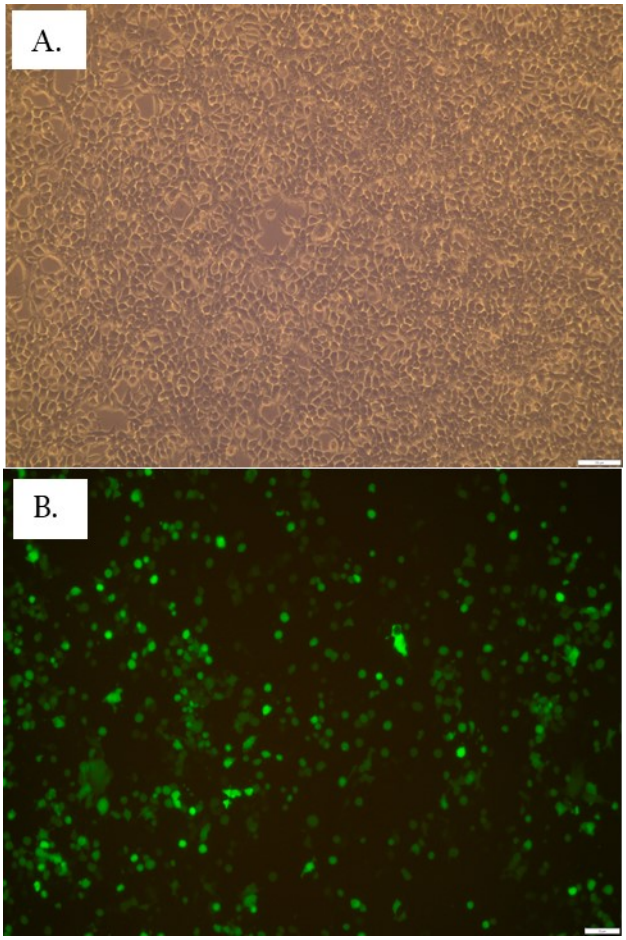

Figure 3. GFP Expression in transfected HEK293T cells. Expression of eGFP in used to determine transfection efficiency of HEK293T cells at 48-hours post-transfection. (A) Control transfected cells showed no GRP expression. (B) Cells transfected with pDUOsh5834.8403 showed high levels of eGFP expression indicating efficient transfections. Cells were visualized at 40x magnification using an inverted fluorescence microscope.

Discussion

The findings of this study underscore the potential of RNA interference (RNAi) as a therapeutic strategy against HIV-1 by targeting two regulatory proteins, Tat and Rev. Tat-targeted and Rev-targeted shRNAs individually demonstrated significant inhibition of their respective targets, resulting in a marked decrease in viral gene expression (Fig 4A). The most interesting outcome of this study was observed with the dual shRNA plasmid, which simultaneously targets both Tat and Rev. Dual shRNA expression produced greater suppression of Tat and Rev activity compared to that of the individual shRNAs (Fig. 4A). A greater shRNA Affect was observed in cells transfected with a dual expression plasmid (Fig. 4B) suggesting a synergistic effect. This synergy likely arises from the compounded inhibition of two pivotal stages in the viral life cycle: transcription and translation, which are crucial for the production and assembly of new virions. The use of dual shRNA expression might also overcome potential gene silencing of one of the two shRNAs in transfecting cells, which likely explains the variability of single shRNA expression treatments (Fig. 4B).

The use of a dual shRNA plasmid also highlights the versatility and robustness of RNAi technology in creating combinatorial therapies. By designing constructs that can target multiple genes simultaneously, RNAi can be tailored to address the complex mechanisms underlying the pathogenicity of HIV-1. This combinatorial approach may overcome some of the limitations associated with single-target therapies, such as the potential for viral escape mutants that can arise when a single viral component is inhibited.

These findings open avenues for future research into the development of RNAi-based therapeutics that can be used in combination with existing antiretroviral drugs. Such combination therapies could potentially lower the viral load more effectively and reduce the likelihood of drug resistance. Moreover, the success of the dual shRNA approach in this study paves the way for exploring other combinations of viral targets, potentially leading to more comprehensive strategies to combat HIV-1.

In conclusion, this study provides evidence for the effectiveness of RNAi in targeting essential HIV-1 regulatory proteins and highlights the enhanced inhibitory effects achieved through a combinatorial approach. The dual shRNA plasmid targeting both Tat and Rev represents a promising strategy for HIV-1 suppression, offering a potential pathway to more effective and durable treatments. Further research and development of RNAi-based therapies could significantly impact the management and treatment of HIV-1, contributing to the broader goal of achieving sustained viral suppression and ultimately, a functional cure.

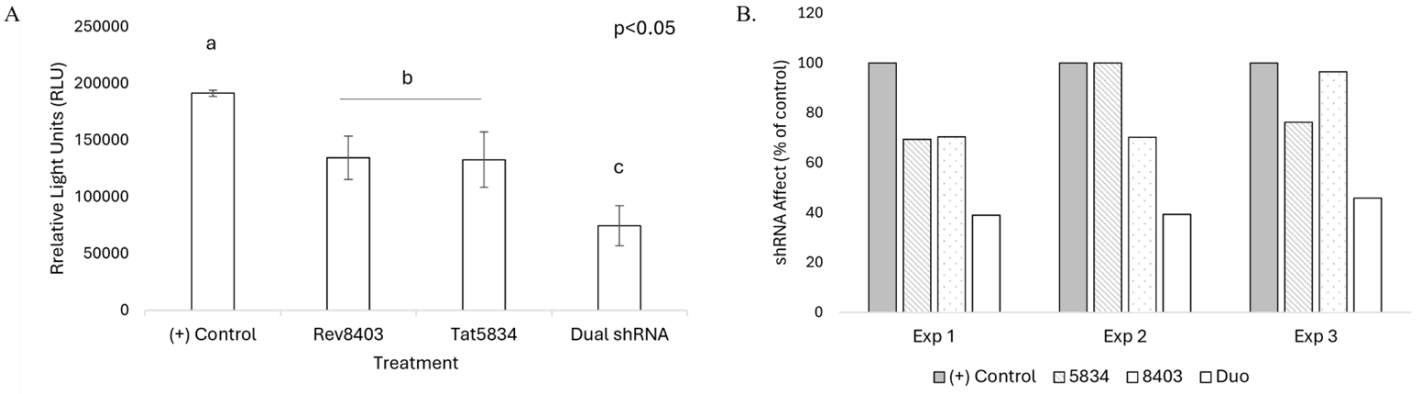

Figure 4. Effect of shRNA expression on HIV-1 Tat and Rev induced luciferase activity. HEK293T cells were co-transfected with 100 ng pNL43.Luc.R$^-$.E$^-$ and 1000 ng of pH1.Tatsh5834, pH1.Revsh8403, pDUOsh5834.8403, or pPCR-Script (+ control). Forty-eight hours post-transfection, the cells were lysed and luciferase expression was measured. (A) A representative plot of the effect of shRNA expression on HIV-1 Tat and Rev mediated luciferase activity. Dual expression of Tat and Rev shRNAs (Dual shRNA) significantly reduced luciferase expression compared to single shRNA expression (Revsh8403 and Tatsh5834). The data represent the mean of three replicate samples taken from two biological replicates. (B) A comparison between three independent experiments showed consistently higher levels of luciferase inhibition of dual shRNA expression compared to single expression of Tat and Rev shRNAs. For this analysis, the shRNA affect was calculated in relation to the (+) control (100%) in each of three experiments and expressed as % of control.

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