

# Promoter Targeting shRNA Suppresses HIV-1 Infection *In vivo* Through Transcriptional Gene Silencing

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Despite prolonged and intensive application, combined antiretroviral therapy cannot eradicate human immunodeficiency virus (HIV)-1 because it is harbored as a latent infection, surviving for long periods of time. Alternative approaches are required to overcome the limitations of current therapy. We have been developing a short interfering RNA (siRNA) gene silencing approach. Certain siRNAs targeting promoter regions of genes induce transcriptional gene silencing. We previously reported substantial transcriptional gene silencing of HIV-1 replication by an siRNA targeting the HIV-1 promoter *in vitro*. In this study, we show that this siRNA, expressed as a short hairpin RNA (shRNA) (shPromA-JRFL) delivered by lentiviral transduction of human peripheral blood mononuclear cells (PBMCs), which are then used to reconstitute NOJ mice, is able to inhibit HIV-1 replication *in vivo*, whereas a three-base mismatched variant (shPromA-M2) does not. In shPromA-JRFL-treated mice, HIV-1 RNA in serum is significantly reduced, and the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> T cells is significantly elevated. Expression levels of the antisense RNA strand inversely correlates with HIV-1 RNA in serum. The silenced HIV-1 can be reactivated by T-cell activation in *ex vivo* cultures. HIV-1 suppression is not due to offtarget effects of shPromA-JRFL. These data provide “proof-of-principle” that an shRNA targeting the HIV-1 promoter is able to suppress HIV-1 replication *in vivo*.

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**Subject Category:** siRNAs, shRNAs, and miRNAs Therapeutic proof-of-concept

## Introduction

Currently available combined antiretroviral therapy has markedly improved both morbidity and mortality associated with human immunodeficiency virus (HIV)-1 infection, reducing the viral load ((VL) HIV-1 RNA in serum) and rescuing CD4<sup>+</sup> T cells from HIV-1 infection.<sup>1–4</sup> However, HIV-1 persists in its proviral form in cellular reservoirs.<sup>5–7</sup> On cessation of even prolonged combined antiretroviral therapy, rapid viral recrudescence occurs in the overwhelming majority of cases.<sup>8,9</sup> Alternative therapeutic approaches are required to overcome these limitations. We have been investigating a transcriptional gene silencing (TGS) approach using short interfering RNAs (siRNAs) gene targeting the promoter region of HIV-1. Unlike siRNA targeting HIV-1 messenger RNA (mRNA), which induces the post-TGS (PTGS) pathway to degrade mRNA in the cytoplasm, we and others have shown that specific siRNAs targeting viral promoter regions can induce TGS within the nucleus.<sup>10–16</sup> TGS has also been demonstrated in an *in vivo* model targeting the promoter of vascular endothelial growth factor (VEGF-A).<sup>17</sup>

Although initial reports demonstrated inhibition of HIV-1 replication by siRNA through PTGS,<sup>18,19</sup> further *in vitro* studies revealed a number of modes of resistance: directly, through rapid development of mutations within<sup>20–22</sup> or near the siRNA targeted region,<sup>23</sup> and indirectly, via mutations in regions separate from the RNA interference targets.<sup>24</sup> Nonetheless, both direct and indirect modes of resistance compromise the efficacy of combinations of siRNAs targeting multiple HIV mRNA regions.<sup>25</sup> Therefore, PTGS approaches appear to have fundamental limitations.

siRNA-induced TGS was originally reported in plants.<sup>26–29</sup> TGS has more recently been observed in certain mammalian cells.<sup>11,30,31</sup> TGS has potential advantages over PTGS when silencing of HIV is the objective. The high mutation rate of HIV-1, due to its nonproof reading reverse transcriptase (RT) and high replication rates, allows rapid adaptation to environmental pressures including the development of resistance or escape mutations.<sup>32,33</sup> As TGS results in marked reduction of HIV-1 transcription through induction of epigenetic modifications in the HIV-1 promoter,<sup>34</sup> production of new viral RNA is limited and the HIV-1 RT enzyme has no substrate on which to act. Therefore, resistance mutations are less likely to develop in a TGS approach.<sup>16</sup> However, TGS approaches may have their own pitfalls. Offtarget effects must be carefully excluded as they have been described with siRNAs or antisense RNA designed to induce TGS.<sup>35</sup> Sequence-specific offtarget effects are difficult to predict and even slight offsetting of target sequences can make substantial changes to the extent of offtarget effects.<sup>36</sup> Furthermore, sequence-nonspecific offtarget effects can be induced by the triggering of interferon (IFN) pathways by double-stranded RNA through endosomal receptors such as Toll-like receptor (TLR)3, TLR7, and TLR8.<sup>37,38</sup>

We have reported sustained, profound, highly specific viral suppression of viral replication by siRNA- and short heparin RNA (shRNA)-induced TGS of HIV-1 and simian immunodeficiency virus in various *in vitro* models, through a mechanism that results in chromatin compaction.<sup>34,39–42</sup> Because HIV-1 has identical long terminal repeats (LTRs) at the 5' and 3' ends of the integrated virus, any promoter-targeted siRNA

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can potentially act through PTGS. With our lead candidate, called PromA, we have found that the contribution of PTGS is limited.<sup>34</sup> In this study, we used a lentiviral delivery system to express the previously described shRNA targeting the HIV-1 promoter region to transduce human PBMCs. We first assessed shRNA-mediated TGS approach using a PBMC infection model *in vitro*. We then demonstrated an antiviral effect of this construct on HIV-1 infection *in vivo* using NOJ mice<sup>43</sup> transplanted with the lentivirus-transduced PBMCs.

## Results

### shRNA targeting the promoter of HIV-1<sub>JRFL</sub> suppresses viral expression in PBMCs obtained from healthy donors

Our previous *in vitro* TGS studies were based on PromA targeting the NF- $\kappa$ B region of the U3 promoter region of HIV-1 (Figure 1a). The humanized NOD/SCID Janus kinase 3 knockout mice model has been developed to use the HIV-1<sub>JRFL</sub> strain.<sup>43</sup> We sequenced the HIV-1<sub>JRFL</sub> promoter region, which demonstrated that there was a one-base mismatch compared with the original sequence targeted by PromA (Figure 1a). Because induction of TGS is sequence specific, and a two-base mismatched siRNA failed to induce effective TGS *in vitro*,<sup>42</sup> we constructed U6 promoter driven-shRNA expression self-inactivated lentivirus vector plasmids with a GFP expression unit (Figure 1b) specifically targeting this region of HIV<sub>JRFL</sub> (shPromA-JRFL), as well as shPromA-M2, a three-base mismatched control, and shPromA-Sc (a scrambled control) (Figure 1b). VSV-G envelope pseudotype lentiviruses expressing each of these constructs were used to transduce human PBMCs. A transduction efficiency of 38.4% for shPromA-JRFL, 31.7% for shPromA-M2, and 34.7% for shPromA-Sc was achieved as assessed by EGFP expression 5 days after transduction (Figure 1c). PBMCs transduced with shPromA-JRFL, but not those transduced with control lentivirus, challenged with HIV-1 *in vitro*, showed significant reduction of HIV-1 *gag* mRNA (Figure 1d).

The detection of *gag* mRNA reflects transcription of unspliced viral RNA. We also investigated whether the transcription of spliced viral RNA was modulated by shPromA-JRFL by measuring levels of spliced-*tat* RNA (Figure 1e). As expected, shPromA-JRFL spliced-*tat* expression was significantly reduced, but with a different kinetic to the suppression of *gag* RNA. This results in a marked difference in the kinetics of the ratio of spliced (*tat*): unspliced (*gag*) RNA in the shPromA-JRFL-treated cultures compared with the control cultures with a peak in the spliced:unspliced ratio at day 7 (Figure 1f). By day 14, the levels of both *gag* and spliced-*tat* RNA are similar in each of the cultures, consistent with loss of effect. Sequence of the virus obtained from the culture supernatant of PBMCs transduced with lenti-shPromA-JRFL at day 14 did not show any mutations in U3 region and, in particular, in the shRNA target sequence. Given that only 38.4% of cells in these bulk cultures were transduced, these results suggest that the elevated HIV-1 replication by day 14, as assessed by both spliced and unspliced viral RNA, is likely due to overgrowth of virus from untransduced cells. Having demonstrated the *in vitro* efficacy of our new construct, we proceeded to *in vivo* experiments using shPromA-M2 as a control, because this three-base mismatched control

is a more rigorous specificity control than the scrambled shPromA sequence.

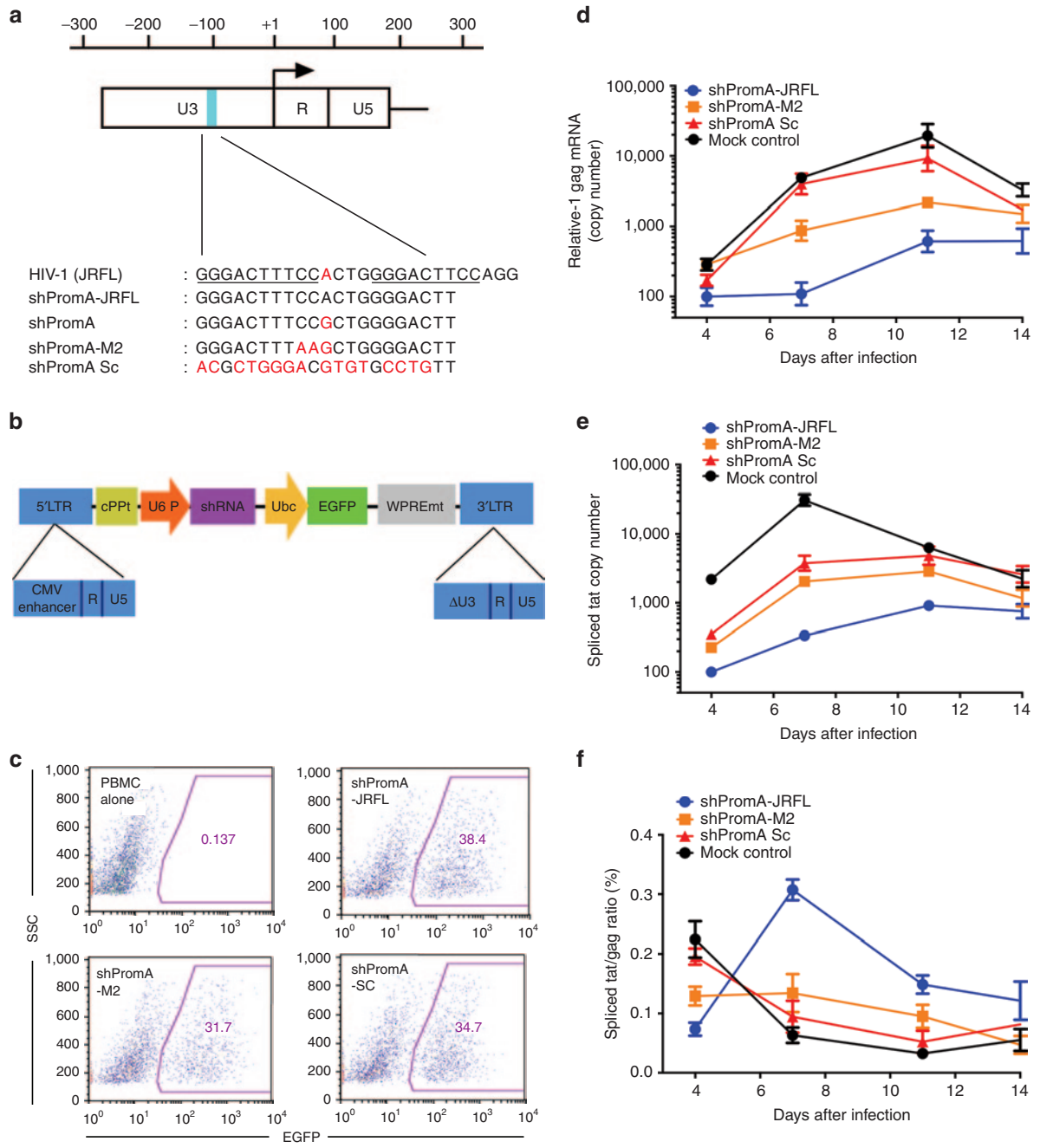
### shPromA-JRFL inhibits HIV-1 replication in a humanized NOJ mouse model

We evaluated the *in vivo* antiviral effect of shPromA-JRFL in a previously established model of acute HIV-1 infection based on the nonobese diabetic (NOD)/SCID/Janus kinase 3 knockout (NOJ) mice reconstituted with human PBMCs and then infected with HIV<sub>JRFL</sub>.<sup>43</sup> First, we transduced healthy human PBMCs with lentivirus-expressing shPromA-JRFL or shPromA-M2. Transduction efficiency before transplantation was 22% for shPromA-JRFL and 25% for shPromA-M2. Seven days later, mice ( $n = 8$  per group) were transplanted with  $1 \times 10^7$  (nonselected) lentivirus-transduced PBMCs per mouse by intraperitoneal injection and the cells allowed to engraft. Five days later, mice were infected by intraperitoneal inoculation of HIV-1<sub>JRFL</sub> (Figure 2a). This is a model of rapidly progressive HIV-1 infection with high VLs, massive CD4<sup>+</sup> T-cell depletion, and profound immunodeficiency occurring within weeks of infection.<sup>43</sup>

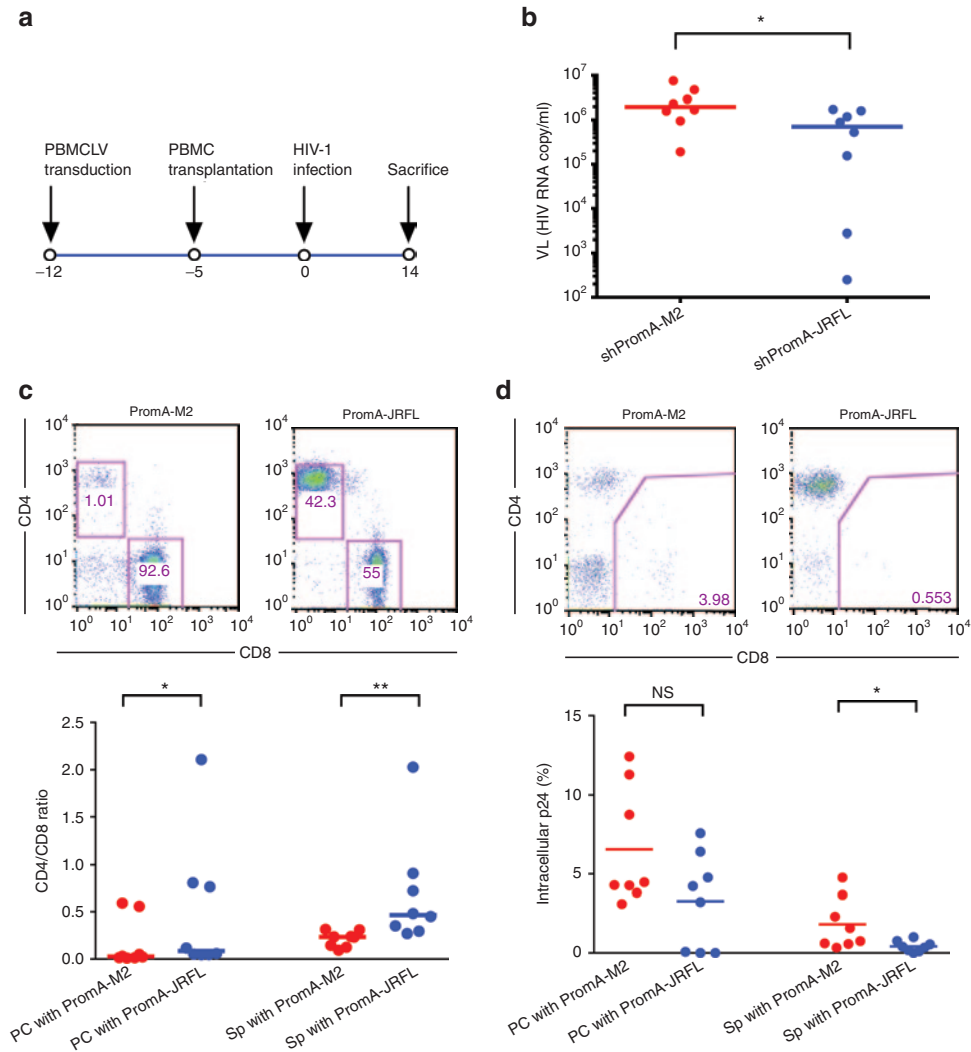
Mononuclear cells were recovered at sacrifice (day 14 after HIV-1 infection) from the peritoneal cavity and the spleen. VL in serum was detected by RT quantitative real-time PCR (RT-qPCR). VL in the mice transplanted with PBMCs expressing shPromA-JRFL was significantly lower ( $P = 0.014$ ) than in shPromA-M2 control mice (Figure 2b). CD4<sup>+</sup> T cells were reduced relative to CD8<sup>+</sup> T cells in shPromA-M2-transplanted mice, whereas the CD4<sup>+</sup> to CD8<sup>+</sup> T-cell ratio was better preserved in mice transplanted with shPromA-JRFL both in the peritoneal cavity ( $P = 0.038$ ) and in the spleen ( $P = 0.002$ ) (Figure 2c). Furthermore, the extent of downregulation of CD4 surface expression is reduced by shPromA-JRFL (Supplementary Figure S1). Thus, shPromA-JRFL appears to protect CD4<sup>+</sup> T cells against HIV-1-mediated depletion and downregulation of CD4 surface expression. By contrast, there was no significant difference in CD8<sup>+</sup> T-cell numbers between the two groups, indicating successful human PBMC engraftment in all the mice (Supplementary Figure S2). Intracellular staining after gating on human CD3<sup>+</sup> CD8<sup>-</sup> spleen cells demonstrated that the percentage of p24-expressing (p24<sup>+</sup>) cells was significantly lower in the mice transplanted with shPromA-JRFL-transduced PBMCs ( $P = 0.014$ ) (Figure 2d).

### Expression levels of the antisense strand of shPromA-JRFL inversely correlated with VL

The above data indicate a reduction in viral replication and relative protection from CD4<sup>+</sup> T-cell destruction, but there was substantial variability in the extent of these effects among the mice within the PromA-JRFL-treated group. Previous observations have suggested that the antisense strand of double-stranded siRNA is responsible for induction of TGS in mammalian cells.<sup>14,39,44</sup> After transduction of the shPromA-JRFL lentivirus, the shRNA expression unit is transcribed from the U6 promoter, by RNA polymerase III, which terminates at a poly(T) motif within this expression unit. The short hairpin loop sequence is then processed by cellular ribonucleases to form mature/processed double-stranded siRNA.<sup>45</sup> We, therefore, quantified the antisense strand of the shPromA-JRFL transcript by real-time PCR, as previously described,<sup>42</sup> to



**Figure 1 Human immunodeficiency virus (HIV)-1 transcription is inhibited by lenti-shPromA-JRFL in peripheral blood mononuclear cells (PBMCs).** (a) Alignment of self-inactivating (SIN) lentivirus vector constructs along with HIV-1<sub>JRFL</sub> target sequences. A map of HIV-1 5' long terminal repeat (LTR) region is illustrated: the blue bar indicates the location of NF- $\kappa$ B binding region; the arrow indicates the HIV-1 transcription start site; red text in the alignment highlights nucleic acids that differ from the HIV-1<sub>JRFL</sub> sequence; numbers indicate the nucleic acid location relative to the transcription start site; and underlined text indicates NF- $\kappa$ B binding region in the HIV-1 promoter. (b) Structure of SIN lentivirus vector. SIN vector consists of central polypurine tract (cPPT), U6 promoter (U6 P), short hairpin RNA (shRNA), ubiquitin C promoter (Ubc), and enhanced green fluorescent protein (EGFP). WPREmt, mutant woodchuck promoter response element, and modified LTR, allow integration but not expression of viral genome. We constructed U6 promoter-driven shRNA expression SIN lentivirus vector plasmid with EGFP expression unit targeting shPromA-JRFL, shPromA-M2 (three-base mismatched control), and shPromA-Sc (scramble control). (c) Expression of EGFP after transduction of lenti-shPromA, shM2, shSc into PBMCs. PBMCs prepared from a healthy donor were stimulated with interleukin-2 for 6 hours, followed by transduction of the lentivirus with a multiplicity of infection of 10. Five days later, EGFP expression was determined by flow cytometric analysis. (d) HIV-1 transcription is inhibited in PBMCs transduced by lenti-shPromA-JRFL.  $2 \times 10^6$  transduced PBMCs were infected with 50 ng HIV-1<sub>JRFL</sub>, as determined by the reverse transcriptase assay. The cell-associated HIV *gag* messenger RNA (mRNA) copy number normalized to 1,000 copies of GAPDH is shown along with time after HIV-1<sub>JRFL</sub> infection. (e) HIV-1 spliced-*tat* expression is modulated in PBMCs transduced with lenti-shPromA-JRFL. Spliced-*tat* mRNA copy number normalized to 1,000,000 copies of GAPDH is shown following after HIV-1<sub>JRFL</sub> infection. (f) The ratio of spliced-*tat* over unspliced HIV-1 mRNA, measured by HIV-1 *gag* mRNA, is shown following HIV-1<sub>JRFL</sub> infection. In panels d, e, and f the mean values and SEM of three independent experiments are plotted.



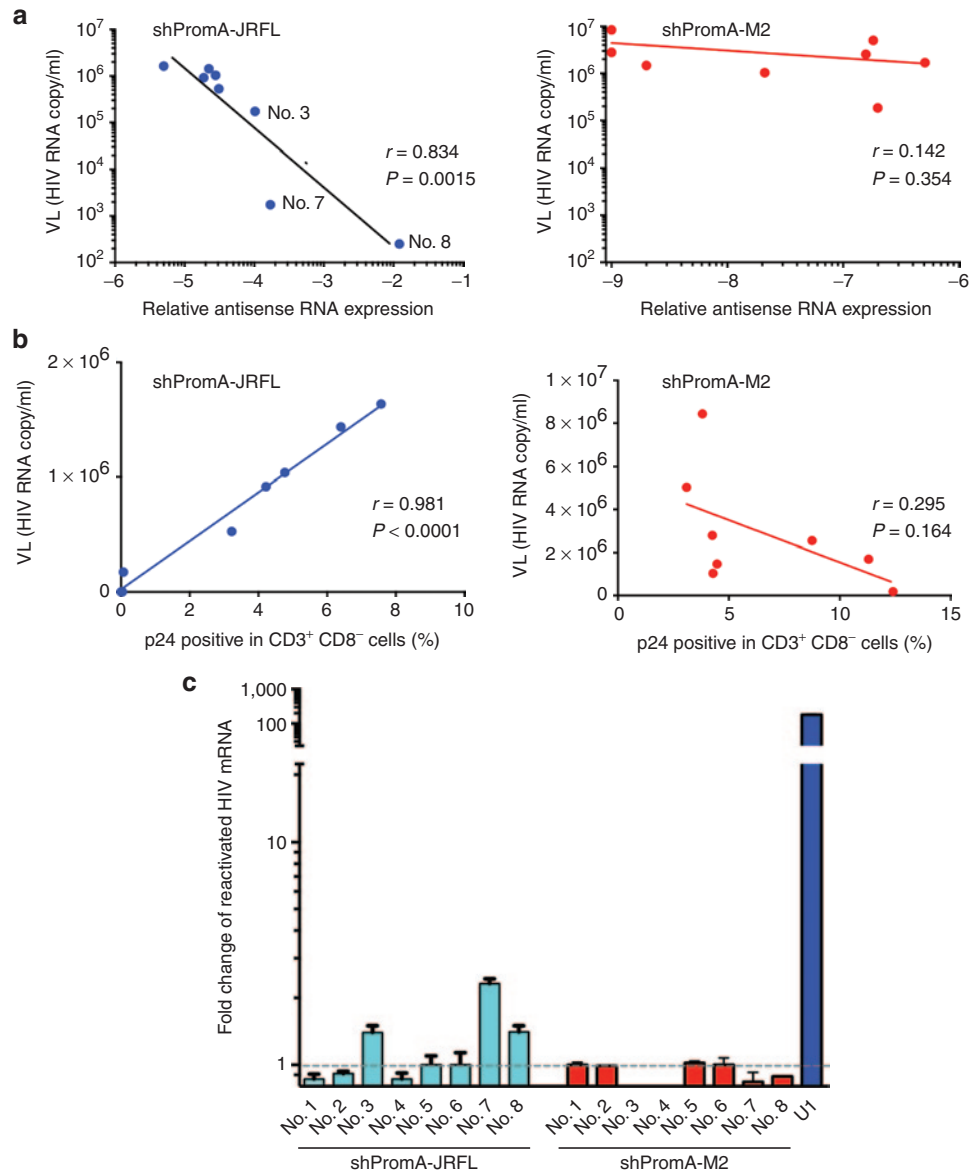
**Figure 2** Transduction with lentivirus-shPromA-JRFL shows antiviral effects in NOJ mouse. (a) Time line for *in vivo* NOJ mouse experiment. LV denotes lentivirus. (b) Amount of viral load (VL) (HIV-1 RNA in serum) in mice with lentivirus-transduced peripheral blood mononuclear cells (PBMCs). Blood samples were collected from mice orbit on day 14 after HIV-1<sub>JRFL</sub> infection. Horizontal bars indicate the medians. \* $P < 0.05$ . (c) Effects on the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> cells in mice with lentivirus-transduced PBMCs. Short bars indicate the medians. \* $P < 0.05$ , \*\* $P < 0.01$ . (d) Effect on intracellular p24-positive cells. Peritoneal cavity cells and splenocytes recovered on day 14 after HIV-1 inoculation were analyzed by flow cytometry. The percentage of p24-positive cells among CD4<sup>+</sup> T cells (gated as mCD45<sup>-</sup> hCD45<sup>+</sup> hCD3<sup>+</sup> hCD8<sup>-</sup>) is shown ( $n = 8$ ). Horizontal bars indicate the medians. \* $P < 0.05$ . NS, not significant; PC, peritoneal cavity; Sp, Spleen.

determine whether the degree of expression and processing of the shRNA constructs impacted the antiviral effects. It was found that the degree of antisense-strand expression had a strong inverse correlation with VL in serum in the mice transplanted with PromA-JRFL-transduced PBMCs ( $r = 0.83$ ;  $P = 0.0015$ ), but not in those transplanted with the control shRNA PromA-M2 (Figure 3a). Similarly, there was an inverse correlation between expression of cell-associated HIV-1 *gag* mRNA in CD4<sup>+</sup> T cells from the spleen and expression antisense strand of the shPromA-JRFL ( $r = 0.84$ ;  $P = 0.0014$ ; Supplementary Figure S3a) and strong linear correlation between VL and expression of cellular-associated HIV-1 *gag* mRNA in CD4 cells ( $r = 0.84$ ;  $P = 0.0014$ ; Supplementary Figure S3b). Consistent with these observations, there was a strong linear correlation between VL and both the percentage of p24-positive CD3<sup>+</sup> CD8<sup>-</sup> cells ( $r = 0.98$ ;  $P < 0.0001$ ;

Figure 3b) and the cell-associated viral mRNA from splenocytes ( $r = 0.84$ ;  $P = 0.0014$ ) in shPromA-JRFL-expressing mice, indicating that serum VL correlates with cellular expression of HIV-1 Gag protein in CD4<sup>+</sup> T cells and *gag* mRNA in splenocytes. These data all point to the fact that the presence of the processed antisense strand of shPromA-JRFL is a strong correlate of inhibition of viral replication.

#### Phorbol myristate acetate, a strong stimulating reagent, reactivates silenced transcription of HIV-1 in *ex vivo* culture

Latent HIV-1 has silenced transcription, which is able to be switched on by strong cellular activating stimuli such as phorbol myristate acetate (PMA).<sup>46</sup> The U1 cell line is a latently infected monocytoid cell line that contains the proviral form of HIV-1, with heterochromatin formation in the viral promoter



**Figure 3 Transcriptional gene silencing (TGS) is induced by lenti-shPromA-JRFL.** (a) Inverse correlation of expression level of the antisense strand of shPromA-JRFL and viral load (VL). Relative antisense RNA expression of shPromA-JRFL was detected by the primer-specific reverse transcriptase–polymerase chain reaction. (b) Linear correlation of intracellular p24 expression level and VL. Intracellular p24 staining in CD3<sup>+</sup> CD8<sup>-</sup> cells obtained from peritoneal cavity was analyzed by flow cytometry and was plotted against VL. (c) Transcriptionally suppressed human immunodeficiency virus (HIV)-1 is reactivated by phorbol myristate acetate (PMA). Splenocytes recovered from day 14 after inoculation of HIV-1<sub>JRFL</sub> were divided into two cultures with or without addition of PMA. After *ex vivo* culture for 24 hours, cellular-associated messenger RNA (mRNA) was extracted for analysis of HIV *gag* mRNA. Substantially increased expression of HIV-1 *gag* mRNA was found after PMA activation in three mice with highly suppressed HIV-1 (Nos. 3, 7, and 8), obtained from shPromA-JRFL treated mice. U1 is a positive control for HIV-1 latently infected cells. The mean values and SEM of three independent experiments are plotted.

region, the 5′LTR. The silenced provirus can be activated by treatment of cells with PMA with concomitant relaxation and opening of the chromatin structure.<sup>47–49</sup> Given that we have previously shown that si/shPromA acts by inducing biochemical changes in histone tails resulting in a heterochromatic structure associated with the 5′LTR, we hypothesized that the silenced HIV-1 induced by shPromA-JRFL would be activated by PMA. We conducted *ex vivo* culture of the splenic CD4<sup>+</sup> T cells from all mice in the shPromA-JRFL–treated group, including the three highly suppressed mice (nos. 3, 7,

and 8, as indicated in Figure 3a). We found that PMA treatment resulted in elevated levels of *gag* mRNA in the PBMCs from the three suppressed mice, but not in those where VL was poorly suppressed. Of note, these were the same mice in which the antisense strand of shPromA-JRFL was poorly expressed (Figure 3c). We also found that PMA treatment did not increase the levels of *gag* mRNA in the PBMCs from the 8 mice treated with shPromA-M2 (Figure 3c). These data are consistent with TGS induced by shPromA-JRFL being responsible for the observed suppression of HIV-1 transcription.

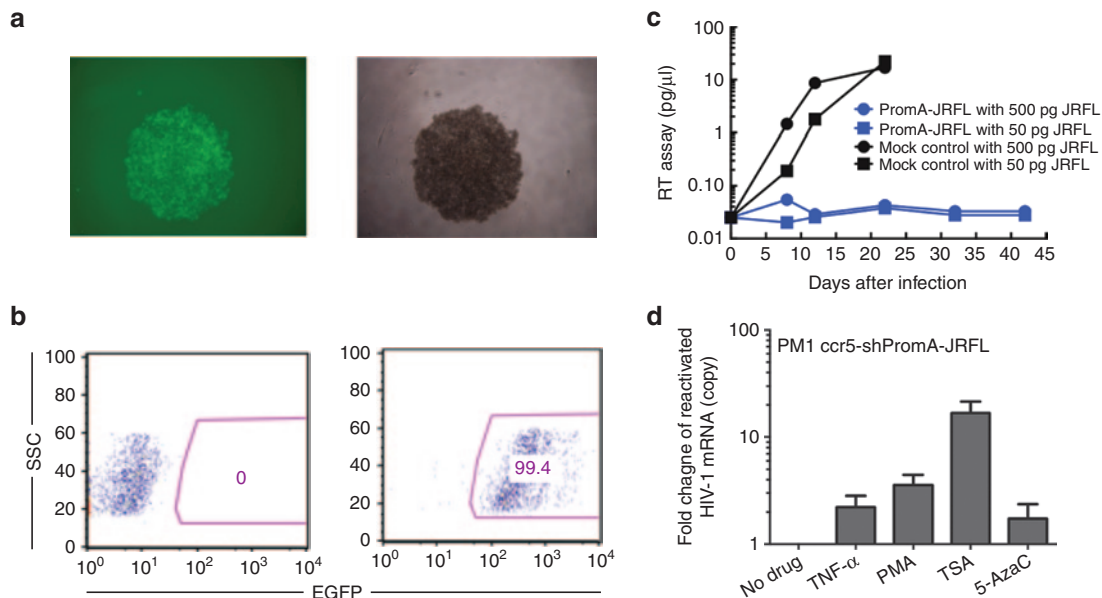
We also confirmed the activation of HIV-1 transcription using an *in vitro* experimental model. We transduced PM1-CCR5 T cells, with lenti-shPromA-JRFL. We conducted limiting dilution of transduced PM1-CCR5 T cells to isolate strongly positive EGFP clonal populations (Figure 4a,b). After confirming expression of EGFP in more than 99% of cells in this clone, (PromA-JRFL No.3), we infected these cells with two concentrations of HIV-1<sub>JRFL</sub> (Figure 4c). We also measured the expression level of the antisense strand of the shPromA-JRFL transcript by RT-qPCR in PM1-CCR5 cells in the presence of ongoing active HIV-1<sub>JRFL</sub> infection. HIV infection did not make a difference to the expression levels of the antisense strand of the shPromA-JRFL transcript (Supplementary Figure S4a,b).<sup>42</sup> After confirming that shPromA-JRFL-expressing PM1-CCR5 cells completely suppressed HIV-1 replication, we then assessed whether activation of the suppressed HIV-1 transcription could be induced by various stimuli, including PMA and the histone deacetylase inhibitor, trichostatin A (Figure 4d). Both stimuli resulted in reactivation of viral replication, with trichostatin A having a greater effect than that of PMA. The powerful effect of trichostatin A in viral reactivation in the presence of shPromA-JRFL strongly suggests that this shRNA is causing viral suppression through TGS, because recruitment of histone deacetylase is a classic mark of TGS. It is interesting that not all activation stimuli result in reactivation of PromA-suppressed infection. GM-CSF stimulation of untransduced U1 cells results in reactivation of latent virus. However, GM-CSF stimulation of U1 cells lentivirally transduced to express shPromA did not result in increased viral replication (Supplementary Figure S5). These data are

concordant with our previous *in vitro* data, demonstrating that siRNA and shPromA cause suppression of HIV-1 replication through TGS.<sup>34,40,41</sup> The data also suggest that TGS mediated through shRNA can be sustained even in the presence of certain cytokines, such as GM-CSF.

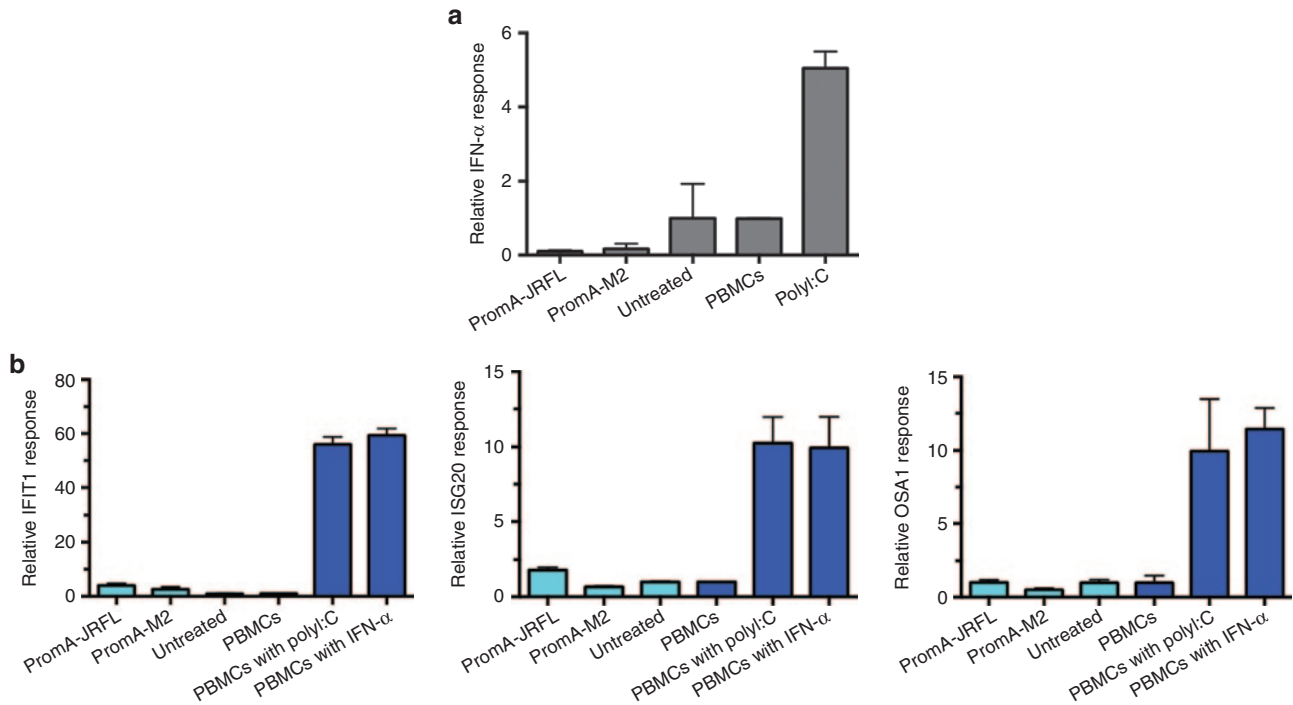
#### TGS induced by shPromA-JRFL was not associated with offtarget effects

Endosomal innate immune receptors, such as TLR3, TLR7, and TLR8, recognize long single- or double-stranded RNAs, triggering type I IFN and IFN-stimulated gene expression that can result in viral suppression by both nonspecific off-target effects and undesirable toxicities.<sup>37,38</sup> We evaluated the extent of induction of IFN- $\alpha$  gene expression using RT-qPCR on splenocytes from shPromA-JRFL, shPromA-M2, and untreated mice. We used polyI:C (polyinosinepolycytosine)-treated PBMCs as a positive control.<sup>50,51</sup> There was no difference in IFN- $\alpha$  expression levels (Figure 5a). Furthermore, by RT-qPCR there was no difference in expression of the IFN- $\alpha$  response genes, OSA1, ISG20, and IFIT1 between groups of mice (Figure 5b), which is consistent with a lack of induction of IFN.

To exclude offtarget effects mediated through the targeting by shPromA-JRFL of other NF- $\kappa$ B binding motifs of host genes as distinct from the NF- $\kappa$ B motif in the HIV-1 LTR, a PCR-based assay was used to assess the expression levels of 86 NF- $\kappa$ B-driven host genes, including IFN- $\alpha$ ,  $\beta$ , and  $\gamma$ . The shPromA-JRFL was not associated with altered expression of NF- $\kappa$ B driven host genes, including the IFN genes (Supplementary Figure S6a,b). These data are concordant



**Figure 4** Transcriptional gene silencing (TGS) is induced by lenti-shPromA-JRFL *in vitro*. (a) PM1-CCR5 cells were transduced with lenti-shPromA with a multiplicity of infection of 10, subjected to limited dilution to isolate EGFP-positive colonies. A fluorescent image of clone PromA-JRFL No. 3 is shown on the left and the corresponding phase contrast image is on the right. (b) Expression of EGFP after expansion of clone PromA-JRFL No.3 as determined by flow cytometric analysis. (c) HIV-1 replication is inhibited in Lenti-shPromA-JRFL-transduced PM1-CCR5 cells. HIV-1 in the culture supernatant was detected by the colorimetric reverse transcriptase (RT) assay. (d) HIV-1 transcription is reactivated from the transcriptionally suppressed PM1-CCR5 cells, transduced with Lenti-shPromA-JRFL. 24 hours after activation with PMA or TSA, cell-associated mRNA was extracted for analysis of HIV *gag* mRNA. Fold change of reactivated HIV-1 *gag* mRNA is shown. 5Aza, 5-azacytidine; PMA, phorbol myristate acetate; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TSA, trichostatin A. The mean values and SEM of three independent experiments are plotted.



**Figure 5** No significant offtarget effects are induced by lenti-shPromA-JRFL. (a) Effect of lentiviral transduction of peripheral blood mononuclear cells (PBMCs) on interferon- $\alpha$  (IFN- $\alpha$ ) expression. Splenocytes were prepared from mice transplanted with lentivirus-transduced PBMCs. Cell-associated messenger RNA (mRNA) was extracted for analysis of IFN- $\alpha$  by reverse transcriptase–polymerase chain reaction (RT-PCR). PolyI:C–treated PBMCs were used as a positive control, indicated in dark blue. (b) Effect of lentiviral transduction of PBMCs on IFN response genes. Cell-associated mRNA was extracted for analysis of three IFN- $\alpha$  response genes (OSA1, ISG20, and IFITM1) by RT-PCR. PolyI:C or IFN- $\alpha$ –treated PBMCs were used as positive controls, these are indicated in dark blue. The mean values and SEM of three independent experiments are plotted.

to our previous analyses of offtarget effects induced by siRNA and shRNA forms of PromA in HeLa and MOLT-4 cell and strongly suggest that the observed HIV-1 suppression is not the result of offtarget effects induced by shPromA-JRFL.<sup>42</sup>

## Discussion

Our previous *in vitro* data based on HeLa or T-cell lines suggested that TGS of HIV-1 can be induced by promoter targeted si/shRNAs through the induction of epigenetic modifications to form heterochromatin structures, which resemble the biochemical modifications of the HIV-1 promoter in latently infected cell lines.<sup>12,13</sup> In this report, we extend our si/shRNA-mediated TGS approach into an *in vivo* NOJ humanized mouse model.<sup>43</sup> Although there are several reports of PTGS mediated by siRNA using *in vivo* humanized mouse models,<sup>50–54</sup> this report demonstrates that HIV-1 gene silencing based on the TGS pathway is possible *in vivo*. Our NOJ model is a model of acute rapidly progressive HIV infection in which massive infection occurs: hCD4/CD8 cell ratio significantly decreases, and high VL is achieved within 14 days of intraperitoneal inoculation of HIV-1<sub>JRFL</sub>.<sup>43</sup> Despite this highly activated, destructive, and rapidly progressive infection with high levels of viral transcription, we were still able to successfully demonstrate a degree of viral suppression using an shRNA that induces transcriptional silencing.

We demonstrated substantial antiviral effects that resulted in significant alterations in a number of surrogate markers of disease progression in the shPromA-JRFL–treated

group, including reduced serum VL, reduced percentage of HIV Gag p24 protein–positive CD3<sup>+</sup> CD8<sup>+</sup> T cells, and an improved ratio of CD4<sup>+</sup>/CD8<sup>+</sup> T cells. Of note, the extent of each of these effects correlated with the extent of expression of the processed shRNA antisense strand. These data also suggest that in mice that showed adequate expression of the antisense strand of shPromA-JRFL in CD4<sup>+</sup> T cells, there was an observable HIV-1 antiviral effect, which we conclude is occurring through TGS. Because the CD4<sup>+</sup> T cells were relatively protected from active HIV-1 infection through shPromA-JRFL–mediated TGS, we could see significant reduction of VL in serum in shPromA-JRFL mice. The data from the *in vitro* PBMC experiments show that shPromA-JRFL has marked effects on production of both spliced and unspliced viral mRNA. Reduction in the production of spliced-*tat* is likely to be important in the effective silencing of latently infected cells, as Tat, through its interaction with the TAR region of the 5′LTR allows efficient upregulation of transcription of long unspliced HIV-1 mRNA.<sup>55–57</sup> Furthermore, the *ex vivo* reactivation of HIV-1 infection by PMA stimulation is consistent with our previously reported observations that siPromA and shPromA constructs result in viral suppression by TGS<sup>40,41</sup> and with other models of HIV-1 latency.<sup>47–49,58</sup> In addition, the *ex vivo* reversal of viral suppression by TNF- $\alpha$  and, in particular, by the histone deacetylase inhibitor, trichostatin A, is consistent with suppression being induced by TGS. We confirmed that shPromA-JRFL did not induce any significant offtarget effects, determined by expression of type

I and II IFNs, IFN response genes and NF- $\kappa$ B-regulated host genes. We also demonstrated that not all activating stimuli reverse this process, for example, the GM-CSF-induced activation of latent HIV-1 in shPromA-transduced U1 cell was inhibited.

This acute human PBMC-NOJ mouse model has been used to demonstrate proof of principle of potential *in vivo* efficacy of shPromA delivered by a retroviral vector, focusing on the relative protection of human CD4<sup>+</sup> T cells against HIV-1 infection. To further this approach, we are investigating the use of newborn NOJ mouse engrafted via intrahepatic injection of human cord blood-derived CD34<sup>+</sup> cells transduced with retroviral constructs expressing shPromA and appropriate controls.<sup>59</sup> This will enable us to evaluate the effect of this approach on engraftment and hematopoietic cell differentiation and reconstitution, and subsequently on HIV-1 infection. An advantage of the CD34<sup>+</sup> NOJ model is that the cell number required in this model is 100-fold lower ( $5 \times 10^4$  CD34<sup>+</sup> cells per mouse) than that of required in the current NOJ mouse model reconstituted by human PBMCs ( $1 \times 10^7$  PBMCs per mouse). The titer of our current lentiviruses is  $\sim 2 \times 10^8$  infectious viral particle per milliliter. Therefore, a higher multiplicity of infection can be achieved to obtain a greater transduction rate, which will potentially provide greater efficacy.

Using the CD34<sup>+</sup> cell-reconstituted NOJ model, we wish to explore a scenario closer to that which we envisage these constructs will be used in human HIV-1 treatment, primarily on cessation of antiretroviral drugs in controlled chronic infection to determine whether lentivirally delivered shPromA constructs can stabilize the viral reservoir on withdrawal of antiretroviral therapy. If the latent viral reservoir could be maintained as effectively silenced by shPromA treatment, these constructs would represent a substantial step forward on the road toward a functional cure, by providing an alternative to the currently proposed eradication strategies than using various viral transcription activating agents, such as histone deacetylase and demethylases.<sup>49,60,61</sup> Rather than activating virus and abolishing infected cells, we propose that constructs such as shPromA could be used to lock HIV-1 into latency maintaining transcriptionally inactive virus even in patients ceasing conventional antiretroviral therapy, thus achieving a prolonged remission or functional cure of HIV-1 infection.

## Materials and methods

**Production of lentivirus.** The construction of lentiviral vector lenti-shPromA-JRFL, lenti-shPromA-M2, and lenti-Sc were previously described.<sup>41</sup> An outline of the construction of self-inactivated lentivirus vector plasmid with GFP expression unit is illustrated in **Figure 1b**. Vesicular stomatitis virus-G (VSV-G) pseudotyped lentiviral vectors were prepared by transduction of plasmid DNA into 293T cells using HilyMax (Dojindo Molecular Technologies, Osaka, Japan), a lipofectamine-based transfection reagent. The resulting virus was concentrated from supernatant as previously described<sup>62,63</sup> and stocks were titrated on 293T cells based on EGFP expression.

**PBMC transduction with lentivirus.** Peripheral blood was collected from healthy volunteers after informed consent was obtained, according to the institutional guidelines approved

by the Faculty of Life Sciences and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan. Healthy donor PBMCs were prepared by standard density gradient centrifugation using Ficoll-Hypaque (VWR, Murarrie, Australia). Cells were cultured in RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), 20% fetal calf serum (R20) in the presence of 20 units/ml of interleukin-2 (Roche Diagnostic, Castle, Hill, Australia) for 7 hours, followed by overnight transduction with either lenti-shPromA-JRFL, lenti-shPromA-Sc, or lenti-shPromA-M2 using a multiplicity of infection of 1.5–2.0. Cells were then cultured in R20 for a further 7 days before transplantation.

**Transplantation of human PBMCs into NOJ mice and HIV-1 infection of mice.** Human PBMC-transplanted NOJ (hu-PBMC-NOJ) mice were generated as described previously.<sup>43</sup> Briefly, NOJ mice were irradiated (1.0 Gy), and bulk lenti-shPromA-JRFL- or lenti-shPromA-M2-transduced PBMCs ( $1 \times 10^7$ ) were resuspended in phosphate-buffered saline (PBS) (0.1 ml) and infused intraperitoneally into each mouse. Seven days after PBMC implantation, a dose of 200 ng of HIV-1<sub>JRFL</sub>, which was determined by HIV-1 p24 antigen ELISA (ZeproMetrix), suspended in 0.1 ml of PBS, was inoculated intraperitoneally into each mouse. On day 14 after HIV-1<sub>JRFL</sub> infection, mice were killed and blood samples were collected from the mouse orbit, and peritoneal cavity and spleen cells were harvested and resuspended in PBS (see **Figure 2a**). All animal experiments were performed according to the guidelines of the Kumamoto University Graduate School of Medical Science.

**RT-PCR analysis and RT assay.** Cellular RNA was extracted using High Pure RNA Tissue Kit (Roche Diagnostic), followed by the RT-PCR analysis as described previously.<sup>39,42</sup> Detection of spliced-*tat* was conducted using the same RT-PCR conditions with the primer set: Tat-F: ATG GAG CCA GTA GAT CCT AGA CTA and Tat-B: ATT CCT TCG GGC CTG TCG using RT-PCR (SensiFAST Probe one-step RT-PCR: Biorline). Both HIV-1 *gag* mRNA and spliced-*tat* mRNA levels were normalized against GAPDH. Colorimetric RT activity (RT assay) in culture supernatants was determined as previously described.<sup>64</sup>

**Flow analysis of CD4<sup>+</sup> CD8<sup>+</sup> T cells and internal p24 staining.** Lymphocyte subsets from human mononuclear cells obtained from the transplanted mice were characterized by flow cytometric analysis as described previously.<sup>43</sup> Briefly, cells were treated with red cell lysing buffer (155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, and 0.1 mM EDTA) to lyse erythrocytes, and single-cell suspensions were prepared in staining medium (PBS with 2% fetal bovine serum and 0.05% sodium azide) and stained with monoclonal antibodies: allophycocyanin (APC)-Cy7-conjugated antimouse CD45 (BD Pharmingen, Kobe, Japan), APC-conjugated anti-hCD4 (Dako, Tokyo, Japan), phycoerythrin-Cy7-conjugated anti-hCD3 (e-Bioscience, Tokyo, Japan), PacificBlue-conjugated anti-hCD8 (BioLegend, Tokyo, Japan), and Pacific Orange-conjugated antihuman CD45 (anti-hCD45) (Invitrogen, Tokyo, Japan). After 30 minutes, cells were washed twice and fixed in PBS with 1% paraformaldehyde for 20 minutes and permeabilized in PBS with 0.1% saponin. After a 10-minute incubation, cells were



stained with phycoerythrin-conjugated anti-HIV-1 p24 monoclonal antibody (Beckman Coulter, Tokyo, Japan) for 30 minutes. All washes and staining procedures were conducted at 4 °C. Following staining, the cells were analyzed on an LSR II flow cytometer (BD Bioscience). Data were analyzed with FlowJo software (Tree Star, Tokyo, Japan).

**Statistical analysis.** RT-PCR analysis and RT assay values are given as mean and SEM. Ratio of CD4<sup>+</sup>/CD8<sup>+</sup> cells and VL were tested for significance using a nonparametric Mann–Whitney *U* test. A *P* value <0.05 was considered statistically significant. All analyses were performed using GraphPad Prism Version 5.0a (Graphpad Software, San Diego, CA).

### Supplementary material

**Figure S1.** Effects of lenti-shPromA-JRFL and lenti-shPromA-M2 on CD4<sup>+</sup> T cells.

**Figure S2.** Effects of lenti-shPromA-JRFL and lenti-shPromA-M2 on CD8<sup>+</sup> T cells.

**Figure S3.** HIV-1 gag HIV mRNA level is inhibited through TGS induced by lenti-shPromA-JRFL.

**Figure S4.** The antisense strand expression level is not altered with HIV-1JRFL infection.

**Figure S5.** Activation of latent HIV-1-infected U1 cells is inhibited by lenti-shPromA.

**Figure S6.** No significant difference in comparison of 86 NF- $\kappa$ B driven genes in PBMCs trasduced with lenti-shPromA.

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