

# Optimized Lentiviral Vectors for HIV Gene Therapy: Multiplexed Expression of Small RNAs and Inclusion of MGMT<sup>P140K</sup> Drug Resistance Gene

Janet Chung<sup>1</sup>, Lisa J Scherer<sup>1</sup>, Angel Gu<sup>2</sup>, Agnes M Gardner<sup>2</sup>, Monica Torres-Coronado<sup>2</sup>, Elizabeth W Epps<sup>2</sup>, David L DiGiusto<sup>2,3</sup> and John J Rossi<sup>1,4</sup>

<sup>1</sup>Department of Molecular and Cell Biology, Beckman Research Institute of City of Hope, Duarte, California, USA; <sup>2</sup>Laboratory for Cellular Medicine, Beckman Research Institute of City of Hope, Duarte, California, USA; <sup>3</sup>Department of Virology, Beckman Research Institute of City of Hope, Duarte, California, USA; <sup>4</sup>Irell and Manella Graduate School of Biological Sciences, Beckman Research Institute of City of Hope, Duarte, California, USA

Gene therapy with hematopoietic stem and progenitor cells is a promising approach to engineering immunity to human immunodeficiency virus (HIV) that may lead to a functional cure for acquired immunodeficiency syndrome (AIDS). In support of this approach, we created lentiviral vectors with an engineered polycistronic platform derived from the endogenous *MCM7* gene to express a diverse set of small antiviral RNAs and a drug resistance MGMT<sup>P140K</sup> marker. Multiple strategies for simultaneous expression of up to five RNA transgenes were tested. The placement and orientation of each transgene and its promoter were important determinants for optimal gene expression. Antiviral RNA expression from the *MCM7* platform with a U1 promoter was sufficient to provide protection from R5-tropic HIV in macrophages and resulted in reduced hematopoietic toxicity compared with constructs expressing RNA from independent RNA polymerase III promoters. The addition of an HIV entry inhibitor and nucleolar TAR RNA decoy did not enhance antiviral potency over constructs that targeted only viral RNA transcripts. We also demonstrated selective enrichment of gene-modified cells *in vivo* using a humanized mouse model. The use of these less toxic, potent anti-HIV vectors expressing a drug selection marker is likely to enhance the *in vivo* efficacy of our stem cell gene therapy approach in treating HIV/AIDS.

Received 20 December 2013; accepted 21 February 2014; advance online publication 1 April 2014. doi:10.1038/mt.2014.32

## INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) causes acquired immunodeficiency syndrome (AIDS) that affects millions of individuals worldwide.<sup>1</sup> The current standard of care is combinatorial antiretroviral therapy which consists of a cocktail of small molecule drugs that target multiple stages of viral replication. Although significantly improving patient survival and quality of life, combinatorial antiretroviral therapy is a lifelong, expensive treatment that is associated with an increased incidence

of cardiovascular disease, lipodystrophy, and neurological complications among other clinical sequelae.<sup>2-6</sup> Furthermore, with the emergence of drug-resistant viral mutants and the persistence of latent viral reservoirs, an alternative and more comprehensive approach to conventional HIV therapy is needed. Gene therapy with HIV-resistant T lymphocytes or hematopoietic stem and progenitor cells (HSPCs) has been proposed as an alternative or supplemental treatment that could lead to a functional cure for AIDS.<sup>7-9</sup> We postulate that generating a lifelong supply of HIV-resistant cells is a critical step in achieving a functional cure for HIV/AIDS.

We are currently developing autologous stem cell transplantation protocols in which gene-modified (HIV-resistant) HSPCs are used to transplant HIV patients in an attempt to provide a persistent source of viral-resistant CD4<sup>+</sup> progeny. Our approach includes the use of small (<200 nucleotides) RNAs to confer HIV resistance to HSPCs and their progeny. These antiviral small RNAs can be grouped into three major classes based on the mechanism of action: small interfering RNAs (siRNAs) to induce posttranscriptional gene silencing; hammerhead ribozymes that catalytically cleave targets with sequence specificity; and nucleolar-localizing RNA decoys or aptamers that sequester targets in a stoichiometric fashion. Similar to traditional combinatorial antiretroviral therapy, RNA-based gene therapy can also be combinatorial with antiviral agents that target multiple stages of viral entry and replication to increase efficacy and reduce the likelihood of viral resistance. Various trigger formats have been used in combinatorial RNA interference (RNAi) approaches, including short hairpin RNAs (shRNAs),<sup>10-12</sup> polycistronic microRNA (miRNA) clusters,<sup>13,14</sup> and long hairpin RNAs,<sup>15</sup> with transgenes expressed from RNA polymerase III (Pol III) and/or RNA polymerase II (Pol II) promoters. Furthermore, multiplexing strategies with different classes of small RNAs to avoid saturation of any processing pathway have also been developed, including our own<sup>16</sup> and others.<sup>17</sup>

We previously reported the use of a clinical vector in which we coexpressed a *tat/rev* siRNA, a nucleolar TAR RNA decoy, and a *CCR5*-targeting ribozyme from independent Pol III promoters to ensure persistent antiviral transgene expression in all hematopoietic cells.<sup>16,18</sup> This combinatorial vector potently inhibited HIV

replication in cord blood HSPC-derived progeny with greatly enhanced efficacy and reduced development of viral resistance relative to vectors with single antiviral RNA constructs.<sup>18</sup> We conducted a pilot clinical trial in four AIDS patients undergoing salvage therapy and autologous stem cell transplantation for lymphoma in which a portion of the HSPCs were modified with this combinatorial vector. We observed rapid engraftment of gene-modified cells with no product-related serious adverse events, demonstrating that the treatment was safe and feasible.<sup>19</sup> Importantly, detection of integrated vector in multiple cell lineages and persistent transgene expression for at least three years following transplantation provide evidence that long-term repopulating cells were modified, and this type of treatment could thus be long lasting.<sup>19,20</sup> However, the frequency of gene marking in peripheral blood mononuclear cells ranged from 0.02 to 0.32% (200–3,200 copies per million cells) and was too low for clinical assessment of antiviral efficacy. It is unclear how much of this low gene marking was the result of the infusion of a 50- to 100-fold excess of unmanipulated HSPCs (for patient safety) versus limitations in the level of transduction of long-term engrafting cells or potential toxicity of the construct to those cells or their progeny. These concerns led us to develop and test second-generation combinatorial lentiviral vectors that expressed lower levels of small RNA transgenes to reduce potential toxicity, while incorporating a drug selection marker for chemical enrichment of gene-modified cells, should the level of transduction of long-term engrafting cells be low.

We used an endogenous polycistronic miRNA cluster located in the intron of the protein encoding *MCM7* gene, previously engineered as a platform to express small RNAs including RNAi triggers and small nucleolar RNAs (snoRNAs) as a strategy to express more physiological levels of anti-HIV small RNA transgenes.<sup>14,21</sup> The *MCM7* platform allows coordinate expression of three antiviral RNAs from a Pol II U1 promoter, eliminating the need for multiple promoters. We previously demonstrated that *MCM7* was a versatile platform for multiplexing different combinations and classes of small RNA antivirals, including *tat/rev*, *rev*, and *tat* RNAi triggers, a nucleolar-localizing TAR RNA decoy (U16TAR), and a nucleolar U5-targeting ribozyme (U16U5RZ) with several combinations strongly inhibiting replication of the NL4-3 strain of HIV in stably transduced CEM T lymphocytes.<sup>21</sup>

In this report, we further extend the capability of the *MCM7* platform in combinatorial gene therapy by incorporating additional antiviral RNAs. We demonstrate expression and processing of up to five antiviral transgenes without concomitant vector toxicity while providing strong antiviral potency in protecting gene-modified HSPC-derived macrophages. In addition, we incorporate a mutant form of the human methylguanine methyltransferase gene (*MGMT*<sup>P140K</sup>) in our constructs to allow prospective chemical selection of gene-modified cells. Endogenous *MGMT* repairs DNA damage from alkylating agents, such as bis-chloroethylnitrosourea (BCNU), but this activity is inhibited in the presence of O<sup>6</sup>-benzylguanine (O<sup>6</sup>-BG). A naturally occurring P140K mutation in *MGMT* (*MGMT*<sup>P140K</sup>) confers resistance to O<sup>6</sup>-BG and thus only cells expressing transgenic *MGMT*<sup>P140K</sup> survive treatment with the combination of O<sup>6</sup>-BG/BCNU. This positive selection strategy increases gene modification allowing evaluation of clinical efficacy. Large animal studies demonstrated

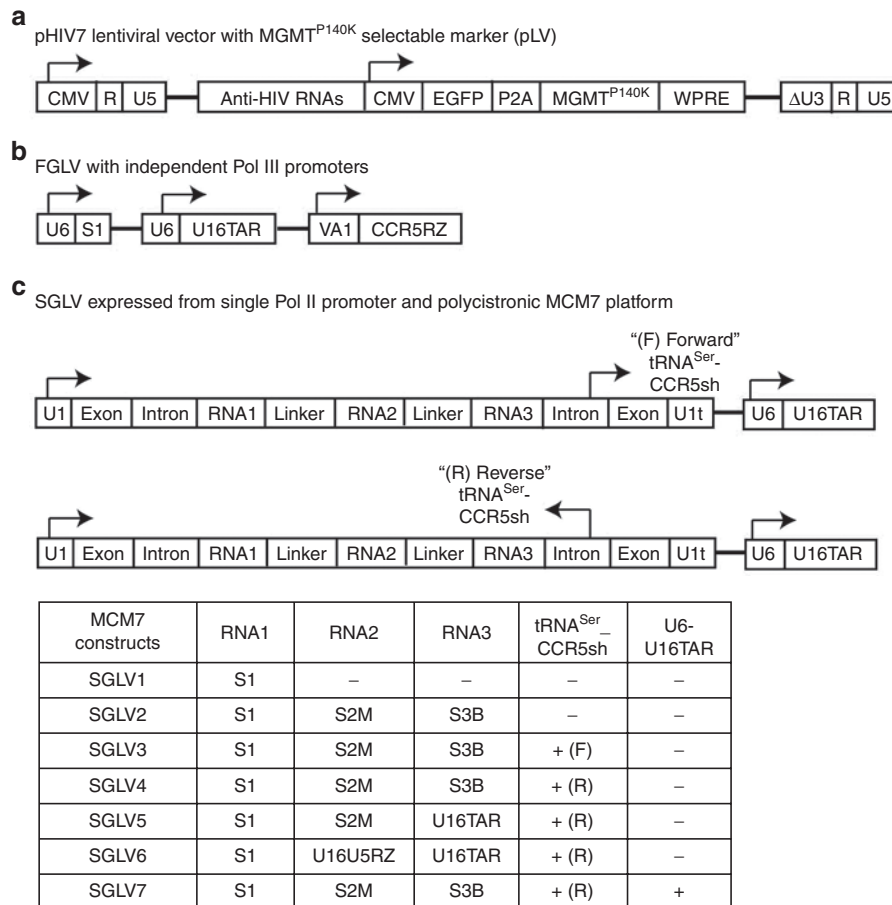
that this selection strategy effectively increases the percentage of gene-modified cells *in vivo* after repeated drug administration.<sup>22–24</sup> We propose that this selection strategy would also work in the human clinical setting and demonstrate the ability of our constructs to protect gene-modified human blood cells during *in vivo* O<sup>6</sup>-BG/BCNU treatment using an immunodeficient (NSG) mouse model. These vector designs have increased antiviral efficacy and reduced toxicity along with the potential to confer selective protection of transduced cells with clinically relevant drugs, providing a foundation for advancing RNA-based stem cell gene therapy into proof-of-concept studies.

## RESULTS

### Lentiviral vector design to incorporate a polycistronic *MCM7* platform and a drug selection marker (*MGMT*<sup>P140K</sup>) for combinatorial RNA-based gene therapy

Lentiviral vectors are efficient gene delivery vehicles with the ability to transduce nondividing cells, such as HSPCs, resulting in long-term expression of the therapeutic transgenes in differentiated progeny. We modified a third-generation, self-inactivating lentiviral vector, pHIV7, that previously demonstrated high efficiency in transducing primary CD4<sup>+</sup> T lymphocytes and HSPCs<sup>25</sup> to also express *MGMT*<sup>P140K</sup> from a CMV promoter (**Figure 1a**). We observed no differences in viral titer and transduction efficiency with inclusion of the *MGMT*<sup>P140K</sup> transgene (data not shown).

Our earliest lentiviral vector used independent Pol III promoters (first-generation lentiviral vector (FGLV), **Figure 1b**) to ensure strong and persistent expression of antiviral small RNA transgenes. Subsequently, we engineered an *MCM7* platform that coexpresses three small RNAs within the polycistronic cluster from a single Pol II U1 promoter (second-generation lentiviral vector, **Figure 1c**) to express small RNAs at more moderate levels to reduce potential vector toxicity. The *MCM7* platform is designed to coexpress three small RNAs within the polycistronic cluster using a single Pol II promoter. Although any Pol II promoter can be utilized to engineer tissue-specific transgene expression in this platform, we selected the U1 promoter for ubiquitous and persistent transgene expression in all hematopoietic cells derived from HSPCs. We previously demonstrated combinations of both si- and snoRNAs can be multiplexed in this format with antiviral functionality.<sup>21</sup> Because the *MCM7* platform expresses small RNAs at lower levels than independent Pol III promoters, we reasoned that we could incorporate additional small RNA transgenes to further enhance antiviral potency without a significant increase in toxicity. We incorporated a *CCR5*-targeting siRNA driven by an independent Pol III transfer RNA Serine promoter (tRNA<sup>Ser</sup>; tRNA<sup>Ser</sup>-*CCR5*sh cassette) in the 3' intron of *MCM7* in both orientations (forward (SGLV3) or reverse (SGLV4), **Figure 1c**) as a viral entry inhibitor. We also introduced a fifth RNA cassette, the nucleolar TAR RNA decoy, driven by the independent Pol III U6 promoter (U6-U16TAR cassette) to increase antiviral potency by inhibiting Tat-dependent viral transcription. This cassette was cloned outside of the *MCM7* transgene to reduce the possibility of promoter interference that could negatively impact gene expression. These novel lentiviral vectors express up to five antiviral small RNAs to block both viral entry and replication of



**Figure 1** Strategy overview on multiplexing small RNAs with different classes of promoters. **(a)** pHIV7 lentiviral vector with MGMT<sup>P140K</sup> selectable marker (pLV). We utilized a third-generation HIV-1–based lentiviral vector (pHIV7) with enhanced green fluorescent protein (EGFP) marker to label gene-modified cells. The chemical resistance MGMT<sup>P140K</sup> marker is coexpressed with EGFP with a self-cleaving P2A peptide with the cytomegalovirus (CMV) promoter. **(b)** First-generation lentiviral vector (FGLV) with small RNAs expressed from independent RNA polymerase III (Pol III) promoters. **(c)** Second-generation lentiviral vector (SGLV) with small RNAs expressed in the MCM7 polycistronic platform with single RNA polymerase II (Pol II) promoter. The naturally occurring microRNA cluster in the intron of the human MCM7 gene was engineered to coexpress different classes of antiviral small RNAs with single Pol II U1 promoter for ubiquitous transgene expression in all hematopoietic cells. Independent Pol III driven RNA cassettes can be incorporated for expression of up to five small RNAs. The tRNA<sup>Ser</sup>-CCR5sh cassette was incorporated into the 3' intron of MCM7 in both orientations (“F” Forward and “R” Reverse) with respect to the parental U1 promoter. The U6-U16TAR cassette was cloned downstream of the U1 termination signal (U1t). Promoters: CMV, cytomegalovirus promoter (Pol II); U6, human small nuclear U6 promoter (Pol III); VA1, adenoviral promoter (Pol III); U1, human small nuclear U1 promoter (Pol II); tRNA<sup>Ser</sup>, human transfer RNA Serine promoter (Pol III). Small RNA transgenes: S1, *tat/rev* siRNA; S2M, *rev* siRNA; S3B *tat*, siRNA; CCR5sh, CCR5-targeting short hairpin RNA; U16TAR, nucleolar TAR RNA decoy; U16U5RZ, nucleolar U5-targeting ribozyme; CCR5RZ, CCR5-targeting ribozyme. HIV, human immunodeficiency virus.

both tropisms of HIV with cassettes driven by both Pol II and III promoters.

### Biological activity and expression optimization of the tRNA<sup>Ser</sup>-CCR5sh cassette in the MCM7 platform

We investigated the use of transfer RNA (tRNA) promoters to express candidate antiviral RNAs due to their small size, ease in multiplexing, and independent regulation of RNA expression. This (Pol III) expression strategy utilizes the endogenous transfer RNA biogenesis pathway to express a primary tRNA-shRNA chimeric transcript which releases the shRNA upon tRNAse Z cleavage<sup>26</sup> for further processing into mature siRNA. In the present constructs, we utilized tRNA<sup>Ser</sup> to express a CCR5 shRNA as an entry inhibitor against R5-tropic HIV. To assess the ability of this construct to downregulate surface CCR5 expression, we

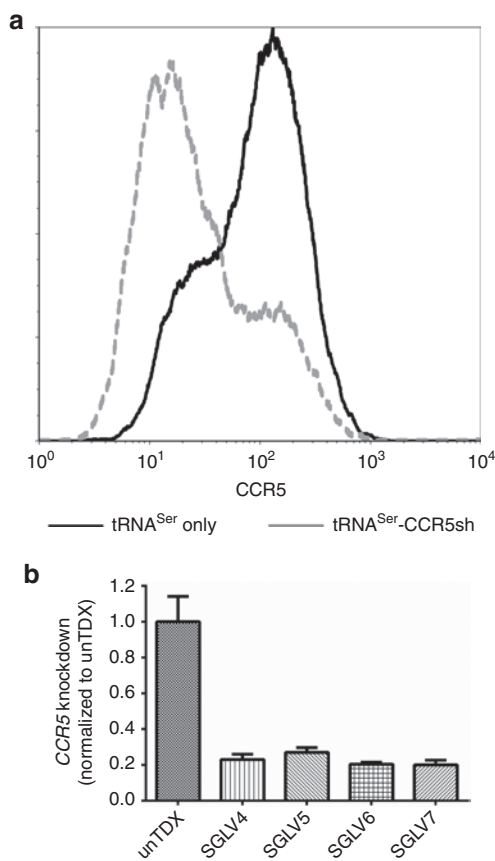
transiently transfected plasmids containing either the promoter sequence only (the endogenous tRNA<sup>Ser</sup> gene) or with the tRNA<sup>Ser</sup>-CCR5sh cassette into CCR5 overexpressing U373-MAGI-CCR5E cells.<sup>27</sup> The reduction in CCR5 expression by flow cytometry was evaluated 72 hours after introduction of the construct. Potent and specific CCR5 knockdown was only observed with the tRNA<sup>Ser</sup>-CCR5sh cassette and not with the (empty) tRNA<sup>Ser</sup> promoter alone (Figure 2a).

We also utilized quantitative reverse transcription polymerase chain reaction (qRT-PCR) to evaluate CCR5 transcript level as the measure of functionality of our tRNA<sup>Ser</sup>-CCR5sh cassette in primary cells where CCR5 protein expression is often difficult to resolve by flow cytometry. We observed 80% downregulation of CCR5 mRNA in primary macrophages derived from *in vitro* differentiation of gene-modified HSPCs. These data demonstrate

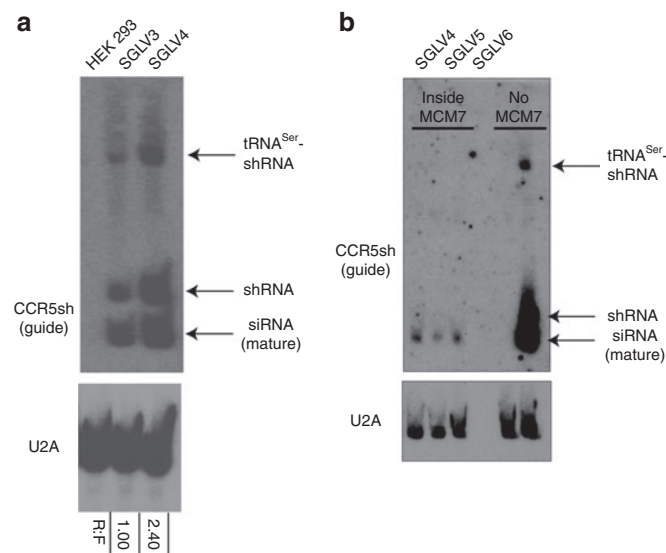
that sufficient *CCR5* siRNA is produced in the context of the MCM7 platform to downregulate gene expression in primary cells (Figure 2b).

To further assess the processing of the tRNA<sup>Ser</sup>-CCR5sh cassette in combination with the other anti-HIV elements, we incorporated this expression unit into the 3' intron of the MCM7 platform ("inside MCM7") in both orientations (forward (SGLV3) and reverse (SGLV4), Figure 1c) with respect to the parental U1 promoter. When transiently transfected into human embryonic kidney (HEK) 293 cells, we observed the reverse orientation (SGLV4) gives 2.4-fold greater transgene expression based on northern blotting with a radioactive probe specific for detecting the guide strand of the *CCR5* shRNA (Figure 3a). Moreover, this analysis further distinguishes products in

various stages of processing including the primary tRNA<sup>Ser</sup>-shRNA chimeric transcript, the released shRNA, and the mature Dicer-processed siRNA sequence capable of gene silencing. The siRNA is the dominate product suggesting efficient processing, but some saturation of the processing pathway due to overexpression from the transient transfection is also evident (Figure 3a). Interestingly, northern blotting analysis shows that pHIV7 containing only the tRNA<sup>Ser</sup>-CCR5sh RNA cassette (no MCM7) drives dramatically higher expression levels in stably transduced CEM T lymphocytes than the above vector where the transgenes are inside the MCM7 3' intron (Figure 3b), even though the vector is present at only one to two integrated copies per cell. These high levels of expression result in the accumulation of incompletely processed tRNA<sup>Ser</sup>-shRNA chimeric transcript and large quantities of shRNAs. Based on these results, we concluded



**Figure 2 Biological activity of tRNA<sup>Ser</sup>-CCR5sh cassette.** (a) Potent *CCR5* knockdown in U373-MAGI-CCR5E cells. Plasmid with only the tRNA<sup>Ser</sup> promoter sequence (solid black line) or with the tRNA<sup>Ser</sup>-CCR5sh cassette (dashed gray line) was transiently transfected into *CCR5* over-expressing U373-MAGI-CCR5E cells<sup>27</sup> with knockdown estimated by flow cytometry 72 hours later. Potent and specific downregulation of surface *CCR5* expression was only observed with the construct containing *CCR5sh*. (b) Potent *CCR5* RNA transcript degradation in gene-modified macrophages. Adult hematopoietic stem and progenitor cells were transduced with the indicated lentiviral vectors, sorted based on enhanced green fluorescent protein expression, and then differentiated into macrophages as described in Material and Methods. *CCR5* transcript knockdown was measured by quantitative reverse transcription polymerase chain reaction with normalization to *GAPDH* housekeeping gene and expressed relative to the untransduced control. tRNA<sup>Ser</sup>-CCR5sh cassette in the context of MCM7 platform induced potent silencing in gene-modified macrophages. tRNA<sup>Ser</sup>, transfer RNA Serine promoter.



**Figure 3 Optimization of the tRNA<sup>Ser</sup>-CCR5sh cassette expression in the MCM7 platform.** (a) Orientation dependence of the tRNA<sup>Ser</sup>-CCR5sh cassette in MCM7. The tRNA<sup>Ser</sup>-CCR5sh cassette was cloned in 3' intron of MCM7 in either the forward (SGLV3) or reverse (SGLV4) orientation then transiently transfected into human embryonic kidney (HEK) 293 cells to evaluate transgene expression and processing by northern blotting. Northern blotting distinguishes products in various steps of processing due to difference in size (tRNA<sup>Ser</sup>-CCR5sh fusion transcript (~130–140 nt) that requires tRNase Z processing; short hairpin RNA (shRNA; ~50–60 nt) that requires Dicer processing; small interfering RNA (siRNA; 20–23 nt) represents the completely processed mature siRNA capable of mediating silencing). In this case, the probe detected the guide strand that mediates *CCR5* silencing. Northern blotting demonstrated that tRNA<sup>Ser</sup>-shRNA cassette is efficiently processed by the RNA interference pathway as the mature siRNA is the predominant product. Furthermore, SGLV4 gives 2.4-fold greater expression in comparison to the opposite orientation after normalization with the loading control U2A RNA. (b) Placement dependence of the tRNA<sup>Ser</sup>-CCR5sh cassette. Placement of tRNA<sup>Ser</sup>-CCR5sh cassette in the lentiviral vector dramatically affects transgene expression in sorted stably expressing CEM T lymphocytes. Although the *CCR5sh* cassette is driven independently from the tRNA<sup>Ser</sup> promoter, the expression was much lower in the context of MCM7 platform ("inside of MCM7") compared to as a separate entity ("No MCM7"). In the latter scenario, overexpression is evident by the presence of unprocessed products (*i.e.*, bands representing tRNA<sup>Ser</sup>-CCR5sh fusion transcript and shRNA). nt, nucleotide; SGLV, second-generation lentiviral vector; tRNA<sup>Ser</sup>, transfer RNA Serine promoter.

that the tRNA<sup>Ser</sup>-CCR5sh cassette in reverse orientation located within the 3' intron of MCM7 platform would provide a more optimal level of siRNA expression.

### Flexible MCM7 platform expresses up to five antiviral small RNAs with efficient processing

Combinatorial therapy has inherent challenges including various types of interference or competition between the individual elements that can negatively impact the potential therapeutic outcome. For example, when a vector encodes multiple RNAi triggers, competition can arise during processing and for incorporation into the RNA-induced silencing complex. To ensure that all the transgenes are expressed and processed into functional forms, we performed northern blotting with CEM T lymphocytes that stably express the transgenes. To ensure that the expression level resembles the physiological condition of one to two copies of integrated vectors in HSPCs, we only sorted transduced populations that were lower than 30% enhanced green fluorescent protein positive (EGFP<sup>+</sup>).

Northern expression analyses indicate that incorporation of additional RNA cassettes such as tRNA<sup>Ser</sup>-CCR5sh did not

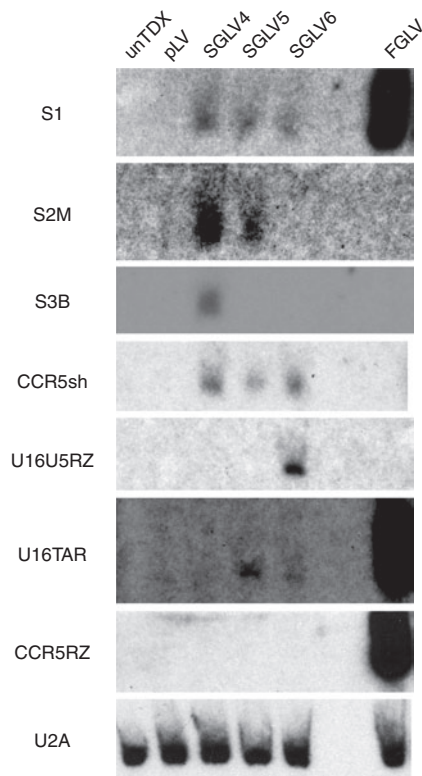
interfere with expression and processing of other transgenes in the MCM7 platform (Figure 4). We also confirmed correct and efficient processing of all RNAi triggers precursors (S1, S2M, S3B, and CCR5sh) into functional mature siRNAs that are 20-23 nucleotides and the small nucleolar RNAs (U16U5RZ and U16TAR) into 132-nucleotide processed products (Figure 4). We did not observe accumulation of pri- and pre-miRNA precursors suggesting that the endogenous RNAi pathway is not saturated and that it is possible to coexpress four siRNAs safely in a combinatorial approach. Therefore, MCM7 serves as a versatile tool in a multiplexing approach with the capacity for incorporating small RNA cassettes driven by both Pol II and Pol III promoters.

Comparing transgene expression levels between combinatorial strategies with two different classes of RNA promoters, SGLVs with the Pol II-driven MCM7 platform express transgenes at lower levels than FGLV with independent Pol III promoters (lanes 3-5 versus lane 6 in Figure 4). While the lower levels resulting from the U1 promoter may reduce toxicity, we questioned whether these levels were sufficient for efficacy. Therefore, we next turned to assessing the anti-HIV activity of the current combinations.

### The MCM7 platform produces sufficient small RNA expression to protect gene-modified cells from R5-tropic HIV

We first assessed antiviral functionality by viral challenge of gene-modified CEM T lymphocytes with R5-tropic JR-FL HIV virus for 42 days, monitoring replication by p24 capsid levels in the culture supernatant (Supplementary Figure S1 and Supplementary Materials and Methods). All the therapeutic constructs (FGLV, SGLV4, SGLV5, and SGLV6) provided long-term protection, with up to a 5-log reduction in p24 capsid production in comparison with pLV and untransduced (unprotected) cells. Notably, SGLV4 and SGLV5 had almost no detectable levels of p24 capsid during this long-term challenge, providing evidence that the MCM7 platform expresses sufficient amount of small RNAs for functionality.

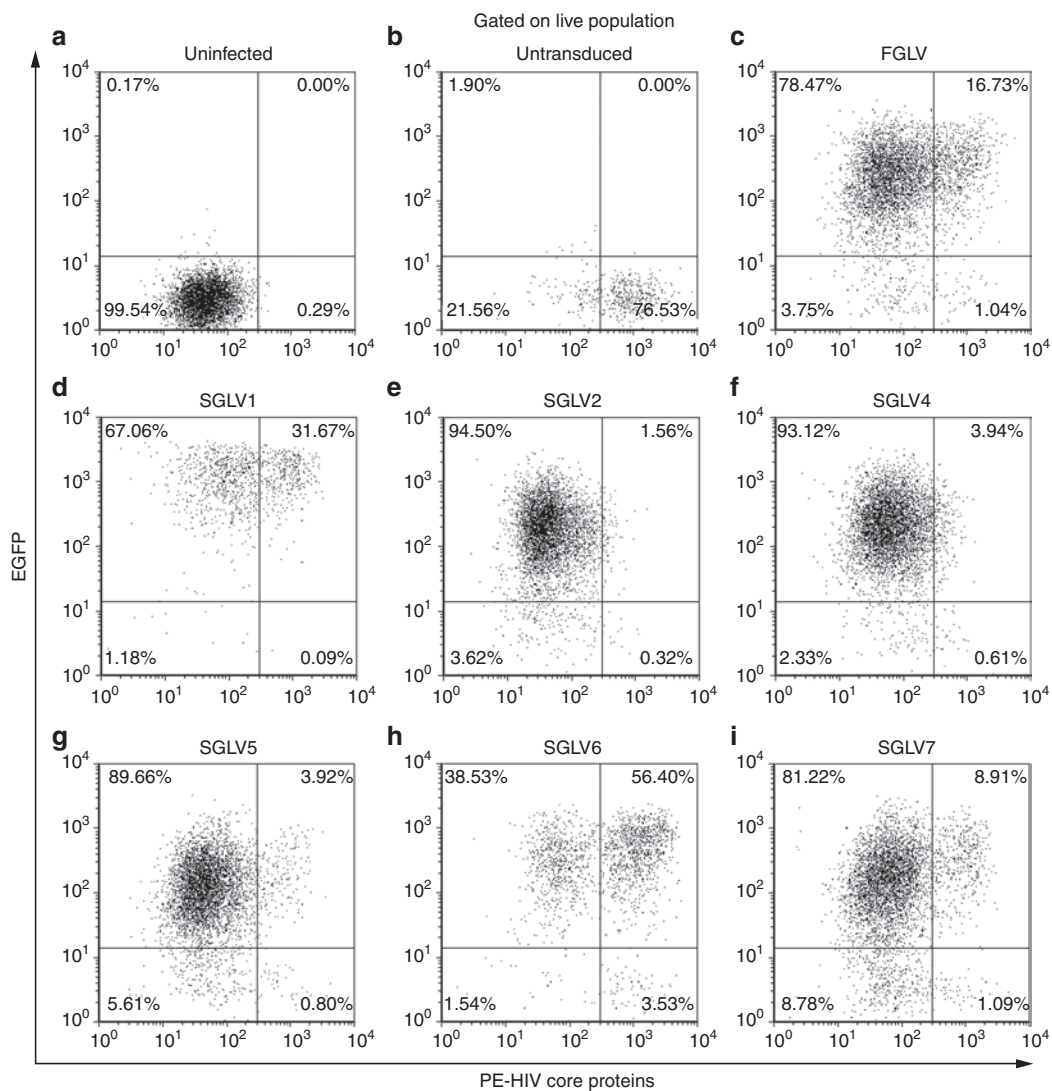
To further demonstrate the feasibility of this stem cell-based approach in protecting gene-modified progenies, we evaluated antiviral potency of our constructs using macrophages derived from HSPCs. Macrophages are myeloid cells particularly suited for demonstrating antiviral potency in HIV challenge assays with vectors expressing CCR5 inhibitors because of the requirement of CCR5 coreceptor usage for infection with R5-tropic virus. We wished to design an assay where adult HSPCs are the substrate for gene modification as they are the eventual clinical target cell population for a stem cell-based gene therapy approach. After transduction, HSPCs can be differentiated into myeloid cells *in vitro*, and the efficacy of the candidate constructs evaluated by challenge with R5-tropic Bal HIV virus. To this end, we developed a novel single cell flow cytometric assay of intracellular staining with an antibody specific to HIV-1 core antigens (55, 39, 33, and 24-kD proteins) to monitor viral replication. The 55-kD protein is the primary precursor, while the 39- and 33-kD proteins are the intermediates of the mature 24-kD core protein.<sup>28</sup> We used uninfected macrophages to establish nonspecific staining and background signal in the flow cytometric assay. Using this novel method, we were able to follow the kinetics of HIV infection on a cellular basis during the 42-day challenge in primary macrophages.



**Figure 4** Northern blot of stably expressing CEM T lymphocytes demonstrates efficient processing and expression of small RNAs. CEM T lymphocytes were transduced with the indicated lentiviruses then sorted by enhanced green fluorescent protein expression to create stably expressing cell lines. Small RNA transgenes were detected by <sup>32</sup>P-labeled probes. U2A small nuclear RNA serves as a loading control. S1, S2M, S3B, and CCR5sh represent fully processed small interfering RNA that are 20-23 nucleotides. U16U5RZ and U16TAR are U16 small nucleolar RNA chimeras that are ~132 nucleotides. CCR5RZ is ~230 nucleotides. Lanes 1: untransduced (unTDX); 2: pLV; 3: SGLV4; 4: SGLV5; 5: SGLV6; 6: FGLV.

Representative intracellular HIV staining results at day 18 (D18) of the viral challenge are shown in **Figure 5**. We observed a 2-log difference in fluorescence intensity comparing nonspecific background staining of uninfected cells (**Figure 5a**) to an actual HIV-infected culture (**Figure 5b**), validating that our novel flow cytometric assay has excellent sensitivity for HIV detection. Furthermore, we found that 76.5% of untransduced (unprotected) macrophages are infected with HIV at D18 (**Figure 5b**) and as high as 95% at later time points (e.g., D28) (**Figure 6**, unTDX trace), providing good dynamic range as a vector screen to distinguish constructs with varying antiviral activities. For example, this flow cytometric assay can distinguish constructs with intermediate protection (31.7% HIV infection in SGLV1 (**Figure 5d**) and 56.4% in SGLV6 (**Figure 5h**)) from constructs with the best

protection (16.7% HIV infection in FGLV (**Figure 5c**); 1.6% in SGLV2 (**Figure 5e**); 3.9% in SGLV4 (**Figure 5f**); 3.9% in SGLV5 (**Figure 5g**); and 8.9% in SGLV7 (**Figure 5i**)). When comparing constructs with the highest antiviral activities, we observed that SGLV2 had better antiviral activity (1.6% infection, **Figure 5e**) compared with small RNAs expressed from independent strong Pol III promoters such as in FGLV (16.7% infection, **Figure 5c**). These results emphasize that the higher levels of small RNA transgene expression are not necessary for anti-HIV potency, as lower RNA expression from the MCM7 platform with a single Pol II U1 promoter is sufficient for functionality. When comparing different RNA combinations in the MCM7 platform (SGLVs), we observed that antiviral protection also increases in constructs containing at least two anti-HIV siRNAs (i.e., SGLV4 and SGLV5). This could



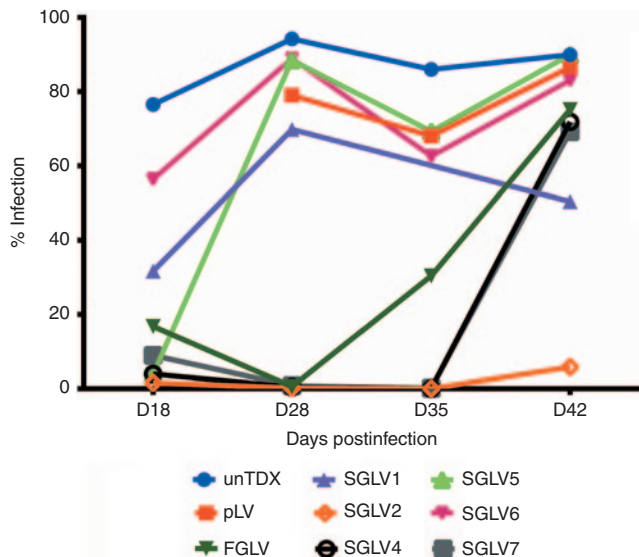
**Figure 5** Intracellular human immunodeficiency virus type 1 (HIV-1) staining demonstrates potent antiviral protection of macrophages derived from gene-modified hematopoietic stem and progenitor cells (HSPCs). Macrophages differentiated from adult HSPCs transduced with indicated lentiviral vectors were challenged with HIV-1 Bal at a multiplicity of infection of 0.01. Viral infection was monitored by intracellular staining by flow cytometry with an antibody specific to HIV-1 core proteins. Data from 18 days postinfection are shown. Background signal for intracellular staining was established with an uninfected control with identical culture and staining protocol. Intracellular staining showed a high degree of infection in unprotected macrophages, with some constructs with intermediate protection while differentiating some with excellent protection. EGFP, enhanced green fluorescent protein; SGLV, second-generation lentiviral vector. **(a)** Untransduced and uninfected, **(b)** untransduced and infected with HIV, **(c-i)** cells transduced with the indicated lentiviral vector and infected with HIV.

be due to the additive nature and efficiency of multiple siRNAs in cleaving HIV targets to inhibit replication.

To our surprise, we observed no added benefit with the inclusion of the tRNA<sup>Ser</sup>-CCR5sh cassette as an entry inhibitor (3.9% in SGLV4, **Figure 5f**) and the nucleolar TAR RNA decoy (8.9% in SGLV7, **Figure 5i**) to the parental SGLV2 construct (1.6% in **Figure 5e**), perhaps because the parental construct already has potent antiviral activity with very low HIV infection. Another measure of potency is the duration of protection from HIV-1 replication. We therefore continue to follow infection for 42 days with the aforementioned intracellular HIV staining methodology to identify construct(s) with persistent protection (**Figure 6**). In this group, we observed an increase in the frequency of infected cells in some constructs over time: SGLV5 (D28) > FGLV (D35) > SGLV4 = SGLV7 (D35), but SGLV2 had no significant increase in viral infection through 42 days of culture. Our long-term results further confirm that overexpression of small RNA is not a guarantee of long-term antiviral protection and that the MCM7 platform can provide lower but sufficient level of RNA expression for functionality.

### Lower small RNA expression reduces potential vector-related toxicity

Having established that sufficient levels of small RNAs are produced from the MCM7 platform for potent antiviral protection, we continued to explore the issue of vector-related toxicity on hematopoietic potential using an *in vitro* colony forming unit (CFU) assay. The CFU assay measures the hematopoietic potential of



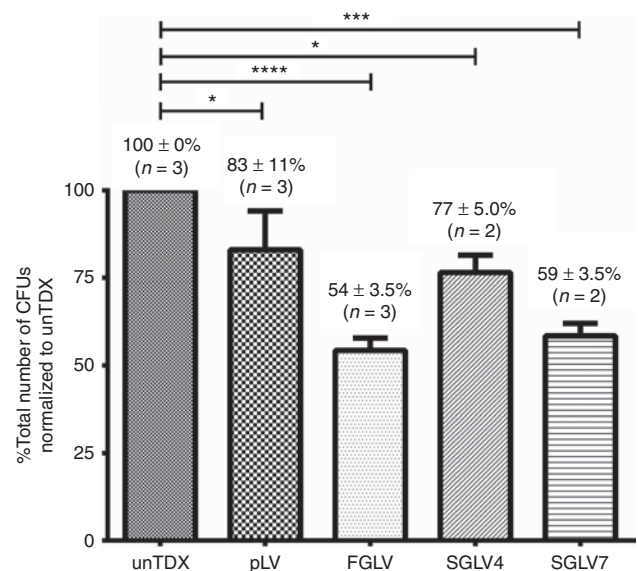
**Figure 6** Kinetics of R5-tropic HIV-1 Bal infection in adult hematopoietic stem and progenitor cell (HSPC) derived macrophages monitored by intracellular HIV staining. Kinetics of HIV-1 Bal infection in macrophages differentiated from gene-modified HSPCs were followed by intracellular HIV staining for a total of 42 days to evaluate long-term protection and viral breakthrough. Overexpression of therapeutic small RNAs with independent Pol III promoters (first-generation lentiviral vector (FGLV)) provided potent protection for up to 28 days but eventual breakthrough. In the long term, SGLV2 provided the longest protection with the incorporation of tRNA<sup>Ser</sup>-CCR5sh (SGLV4) and U6-U16TAR (SGLV7) cassettes less optimal. D, day; HIV, human immunodeficiency virus; SGLV, second-generation lentiviral vector; tRNA<sup>Ser</sup>, transfer RNA Serine promoter.

uni- and multipotent myelo-erythroid progenitors and therefore can be used to assess toxicity of multiplexed RNA expression in gene-modified HSPCs. We normalized the total number of CFUs to the untransduced control with respect to each donor to account for differences in hematopoietic potential between donors.

We found that the transduction process for gene modification with the empty vector had minimal impact on hematopoietic potential ( $83 \pm 11\%$  for pLV, **Figure 7**), which is relevant for clinical translation of stem cell-based gene therapy. In contrast, the levels of therapeutic small RNA expressed from independent strong Pol III promoters in FGLV had a negative impact on hematopoietic potential ( $54 \pm 4\%$ , **Figure 7**). However, when the small RNA expression is lowered with the MCM7 platform (e.g., SGLV4), we observed a recovery of hematopoietic potential to a level similar to that of the empty vector ( $77 \pm 5$  versus  $83 \pm 5\%$ , respectively, **Figure 7**). Surprisingly, incorporating the U6-U16TAR cassette expressing the nucleolar TAR RNA decoy (SGLV7) created a sharp decline in CFU formation (from  $77 \pm 5$  to  $59 \pm 4\%$ , **Figure 7**). It is therefore possible that the U6-U16TAR cassette alone may be responsible for the reduction in hematopoietic potential with FGLV observed in this assay. Further experiments are required to establish the relative roles of higher overall small RNA levels versus the specific effect of the U6-U16TAR moiety on the loss CFU potential observed with FGLV.

### *In vivo* O<sup>6</sup>-BG/BCNU drug selection allows for enrichment of gene-modified cells in humanized NSG mice

One of the current hurdles in stem cell-based gene therapy for HIV is the low frequency of gene-modified cells typically generated by



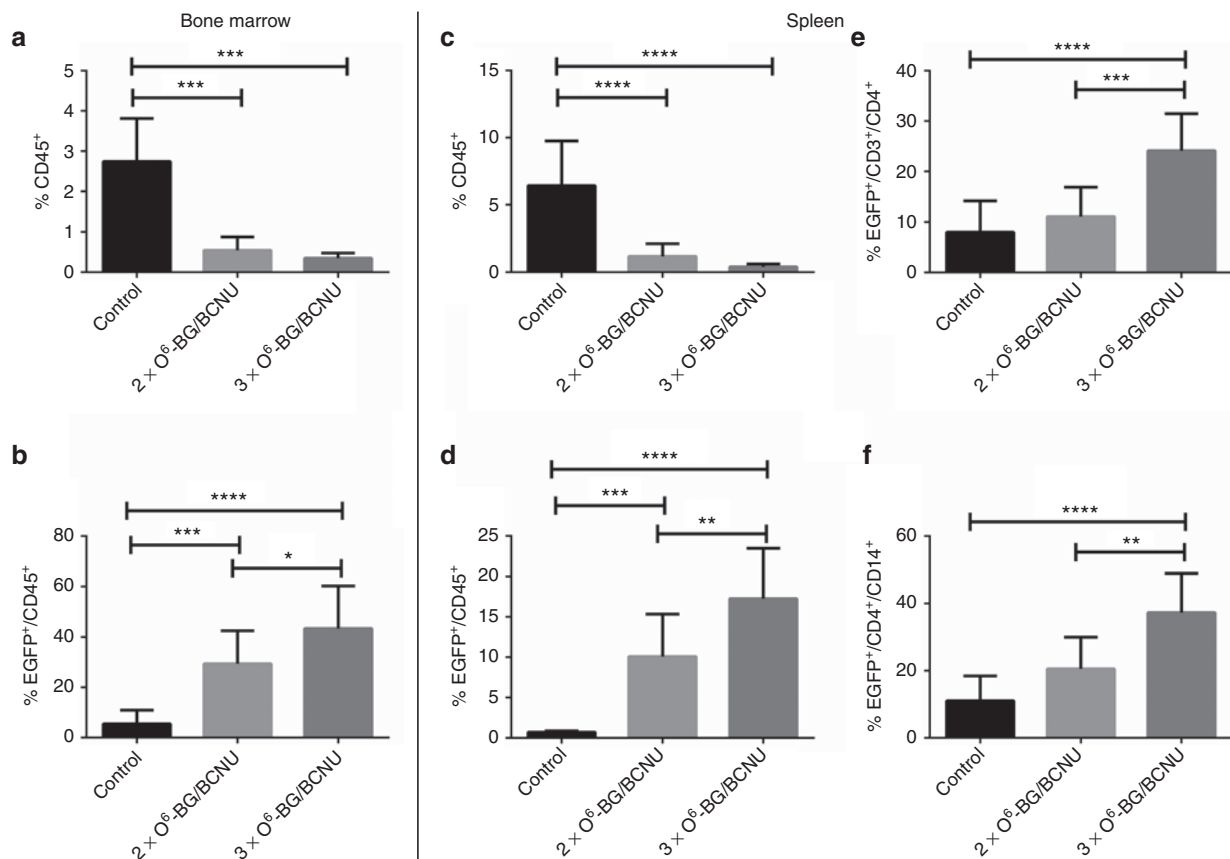
**Figure 7** *In vitro* colony forming unit (CFU) assay to identify vector toxicity on hematopoietic potential. Transduced hematopoietic stem and progenitor cells were sorted on CD34<sup>+</sup>/EGFP expression after expansion with SR1. A total of 500 sorted cells per sample were plated on methylcellulose medium in triplicate with number of colonies counted 12–13 days later. The absolute number of CFUs was normalized to the respective donor to account for differences in hematopoietic potential in donor viability. Results represent data from at least two independent donors with significant results shown. \* $P \leq 0.05$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ . EGFP, enhanced green fluorescent protein.

current *in vivo* protocols, preventing clinical assessment of antiviral efficacy. Therefore, we wished to include a gene in our second-generation lentiviral vectors that confers a survival advantage to a clinically relevant drug in AIDS-related lymphoma patients, thereby increasing the frequency of HIV-resistant cells *in vivo*. The endogenous MGMT enzyme is responsible for repairing DNA damage caused by alkylating agents such as BCNU. O<sup>6</sup>-BG deactivates endogenous MGMT so that cells cannot repair BCNU-induced DNA damage resulting in cell death. However, gene-modified cells that express a modified MGMT (MGMT<sup>P140K</sup>) are not sensitive to O<sup>6</sup>-BG treatment and therefore can repair DNA damage from BCNU treatment and survive. The net result of the O<sup>6</sup>-BG/BCNU selection is increased frequency of gene-modified cells. Therefore, we included this drug resistance gene in our anti-HIV constructs and developed a protocol to test for enrichment of gene-modified HSPC and CD4<sup>+</sup> progeny *in vivo*.

In order to establish that a sufficient level of MGMT<sup>P140K</sup> was expressed in the relevant cell types, HSPCs were transduced with an MGMT<sup>P140K</sup>-expressing vector (FGLV) and used to transplant immunodeficient NSG mice as described in Materials and Methods. Animals were treated with O<sup>6</sup>-BG and BCNU at the 7<sup>th</sup>

and 8<sup>th</sup> weeks (2× treatment cohort) or at the 7<sup>th</sup>, 8<sup>th</sup>, and 9<sup>th</sup> weeks (3× treatment cohort) following transplantation. Two or three weeks after completion of the O<sup>6</sup>-BG/BCNU treatment (*i.e.*, 11 weeks following transplantation), animals were necropsied, and the level of engraftment and frequency of gene-modified cells in the spleen and bone marrow were evaluated.

Our results demonstrate that engraftment of the bone marrow and spleen with human (CD45<sup>+</sup>) cells was significantly reduced in treated animal cohorts relative to the control cohort ( $P < 0.001$ ) (Figure 8a,c, respectively), but the frequency of EGFP<sup>+</sup>/CD45<sup>+</sup> cells in the bone marrow and spleen was enriched 10- and 15-fold in the 2× and 3× treated cohorts, respectively (Figure 8b,d). The average frequency of EGFP<sup>+</sup>/CD3<sup>+</sup>/CD4<sup>+</sup> T lymphocytes in the spleen increased 3-fold only when O<sup>6</sup>-BG/BCNU treatment was performed three times (Figure 8e). Similarly, EGFP<sup>+</sup>/CD4<sup>+</sup>/CD14<sup>+</sup> monocytes were increased 3-fold in the spleens of mice following three O<sup>6</sup>-BG/BCNU treatments (Figure 8f). No deficiencies in lineage development were noted in the drug treated mice (data not shown). These data demonstrate that selective enrichment of HIV-target cells occurred *in vivo* and thus may allow for posttransplant increase in the frequency of HIV-resistant



**Figure 8** *In vivo* drug selection enhances the frequency of gene-modified cells in the bone marrow and spleen of humanized NSG mice expressing MGMT<sup>P140K</sup>. Analysis of NSG mice transplanted with gene-modified hematopoietic stem and progenitor cells (HSPCs) expressing MGMT<sup>P140K</sup> and treated with two or three doses of O<sup>6</sup>-benzylguanine (O<sup>6</sup>-BG)/bis-chloroethylnitrosourea (BCNU) as described in Material and Methods. Each animal received 1 × 10<sup>6</sup> HSPCs following transduction with FGLV at the date of transplantation and 20 μg of Fc/IL-7 protein weekly for 11 weeks. (a) Frequency of CD45<sup>+</sup> cells in the bone marrow of treated mice. (b) Frequency of CD45<sup>+</sup>/EGFP<sup>+</sup> cells in the bone marrow of treated mice. (c) Frequency of CD45<sup>+</sup> cells in the spleens of treated mice. (d) Frequency of CD45<sup>+</sup>/EGFP<sup>+</sup> cells in the spleen of treated mice. (e) Frequency of CD3<sup>+</sup>/CD4<sup>+</sup>/EGFP<sup>+</sup> gene-modified T lymphocytes in the spleen of treated mice (gated on CD45<sup>+</sup> population). (f) Frequency of CD14<sup>+</sup>/CD4<sup>+</sup>/EGFP<sup>+</sup> gene-modified monocytes in the spleen of treated mice (gated on CD45<sup>+</sup> population). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . EGFP, enhanced green fluorescent protein; IL, interleukin.



cells in patients where engraftment of gene modified cells may be suboptimal.

## DISCUSSION

Gene therapy with HIV-resistant HSPCs holds great promise in achieving a functional cure for HIV/AIDS. Among the various avenues of engineering HIV immunity, small RNA agents offer advantages over antiviral proteins due to their lower potential immunogenicity. Similar to combinatorial antiretroviral therapy, multiplexed RNA-based approaches inhibit viral entry and replication via multiple mechanisms and targets to enhance efficacy and to reduce the potential for viral escape. Our previous studies demonstrated that RNA-based gene therapy can be durable with detection of integrated vector in multiple differentiated cell lineages and persistent expression of small RNA transgenes three years after transplantation.<sup>19,20</sup> However, the low frequency of gene-modified cells *in vivo* prevented clinical assessment of efficacy. Therefore, improvements in vector design and prospective enrichment of gene-modified cells are likely needed to advance RNA-based stem cell gene therapy and demonstrate proof of concept in controlled clinical trials.

We recently engineered an endogenous miRNA cluster located in an intron of the protein encoding *MCM7* gene as an improved platform for providing more physiological amounts of therapeutic small RNAs to reduce potential vector-related toxicity. We demonstrate here that *MCM7* is a flexible and versatile platform for coexpressing combinations of up to three si- and snoRNAs and can support additional independent RNA cassettes (both inside and outside of *MCM7*) for a total of five small RNAs. To our knowledge, this is the first example of multiplexing both classes of RNA promoters in a combinatorial approach. We observed expression and complete processing of all small RNA transgenes into functional forms without saturation of processing pathways or promoter interference that could negatively impact transcription. Interestingly, although the tRNA<sup>Ser</sup>-CCR5sh cassette is expressed at high levels in the pLV without *MCM7*, its expression is much weaker in the context of *MCM7*. This result may be related to the requirement for splicing of the intronic *MCM7* cluster prior to transgene expression. Moreover, expression is also related to orientation of the cassette in *MCM7*, highlighting the importance of optimizing placement in multiplexing strategies.

The level of small RNA expression from the *MCM7* platform driven by a single Pol II U1 promoter is much weaker compared to amounts driven by independent Pol III promoters. Nonetheless, we established that sufficient amounts of antiviral small RNA were produced from the *MCM7* platform to protect gene-modified CEM T lymphocytes and primary macrophages derived from gene-modified HSPCs from R5-tropic HIV, with the duration and level of protection highly dependent on the RNA combination. This suggests that the optimal level of RNA expression is more important than achieving maximal levels of expression for a given modality. It is likely that the overall amount of small RNA processing that can occur in a cell at any given time is limited. In theory, because of the catalytic nature of siRNAs and ribozymes in turning over multiple substrates, it should take less RNA to achieve the same therapeutic effect compared to agents that sequester their target in stoichiometric ratio such as TAR RNA decoys.

Our results show that using multiple siRNAs in the *MCM7* platform provides better protection against HIV, *e.g.*, three siRNAs targeting *tat* and *rev* (SGLV2) are better than a single *tat/rev* siRNA (SGLV1). This could be due to the additive nature and efficiency of multiple siRNAs in cleaving HIV targets, while increasing the selective pressure against viral escape. Interestingly, the addition of two other potent anti-HIV RNAs, tRNA<sup>Ser</sup>-CCR5sh entry inhibitor and a U6-driven nucleolar TAR RNA decoy (SGLV4 and SGLV7, respectively), to the parental triple siRNA construct (SGLV2) did not provide additional benefit in inhibiting viral replication or preventing viral breakthrough. In fact, the additional small RNA cassettes actually negatively impacted potency with the observation of viral breakthrough at 35 days with the parental construct observed limited breakthrough through the 42-day culture. It is unclear why addition of the entry inhibitor and the nucleolar TAR RNA decoy was less optimal in inhibiting HIV, although it is possible that *CCR5* siRNA may compete with other anti-HIV siRNAs (*i.e.*, S1, S2M, and S3B siRNAs) for RNA-induced silencing complex factors in gene silencing; if so, then using the forward orientation of the tRNA<sup>Ser</sup>-CCR5sh inside *MCM7* could alleviate competition. Alternatively, the *CCR5* target or shRNA format may be responsible. Furthermore, TAR RNA has been reported as a pri-miRNA that is processed into functional miRNAs<sup>29</sup> and a potent binder to TRBP (HIV-1 TAR RNA binding protein) within the endogenous RNA-induced silencing complex that negatively impacts RNAi pathway. The newly discovered toxicity associated with overexpression of the TAR RNA decoy (see below) could implicate selection of cells with overall less RNA expression translating into less optimal antiviral protection.

We performed an *in vitro* CFU assay to identify any vector-related myelo-erythroid toxicity. Our data demonstrate that the *MCM7* platform has the capacity to safely express four siRNAs with complete processing without reducing *in vitro* hematopoietic potential of gene-modified HSPCs. Surprisingly, we observed a reduction of CFU potential when incorporating the U6-U16TAR cassette, which is the first report of toxicity related to overexpression of RNA decoys. It is possible that the U6-U16TAR cassette is responsible for the observed toxicity in FGLV, but further experiments including assessment of each individual Pol III RNA cassette on CFU potential are likely required. Additional studies may be needed to assess potential toxicity in other (lymphoid) cell populations not addressed in these *in vitro* assays.

Increasing the frequency of gene-modified cells *in vivo* may be required to achieve a clinically meaningful antiviral effect in cases where engraftment of gene-modified cells is low. Therefore, we incorporated a drug resistance gene (*MGMT*<sup>P140K</sup>) in our improved lentiviral vector to allow for *in vivo* enrichment of gene-modified cells using alkylating agents. Our results demonstrate the feasibility of this approach in increasing the frequency of gene-modified CD4<sup>+</sup> cells using *in vivo* treatment with levels of BCNU similar to those used in a non-human primate model of HIV gene therapy.<sup>30</sup>

In summary, combinatorial gene therapy approaches are often most effective when targeting multiple stages of viral replication. We present here the improved *MCM7* platform with enhanced flexibility, increased safety with sufficient level of transgene expression for long-term inhibition of HIV in HSPC-derived

macrophages. We demonstrate that more is not always better but rather a balance between transgene expression, mechanism of action, and target choice is required for optimizing the combinatorial approach. With the improvement in vector efficacy and strategies to increase the frequency of HIV-resistant cells, we hope to achieve sufficient gene marking and antiviral potency to move closer to a functional cure of HIV.

## MATERIALS AND METHODS

**Generation of improved second-generation lentiviral vectors with polycistronic MCM7 platform and selectable MGMT<sup>P140K</sup> marker.** The MGMT<sup>P140K</sup> transgene was coexpressed with the EGFP marker from a polycistronic message utilizing a self-cleaving P2A peptide sequence from the CMV promoter. The MGMT<sup>P140K</sup> gene was first PCR amplified from pRSC-SMPGW2 plasmid,<sup>24</sup> using the following primers (forward: 5'-GGG TCT AGA ATG GAC AAG GAT TGT GAA ATG AAA CGC-3' and reverse: 5'-GGG GAA TTC CGT ACG TCA GTT TCG GCC AGC AGG CG-3') flanked by *XbaI* and *EcoRI* sites. The fragment was digested with appropriate enzymes and then subcloned into *NheI* and *EcoRI* sites of the pFUG-P2A-WPRE vector (Burnett J, Unpublished data) just downstream of the P2A peptide sequence to generate pFUG-P2A-MGMT<sup>P140K</sup>-WPRE. The P2A-MGMT<sup>P140K</sup> fragment was then excised by *BsrGI* and *BsiWI* digestion and subcloned into the *BsrGI* site of pHIV7-GFP vector<sup>25</sup> to generate a modified lentiviral vector pHIV7-GFP-P2A-MGMT<sup>P140K</sup>. The same strategy was utilized to introduce the P2A-MGMT<sup>P140K</sup> fragment into FGLV<sup>18</sup> to generate the modified lentiviral vector suitable for drug selection.

The CCR5-targeting siRNA is expressed from the Pol III tRNA<sup>Ser</sup> promoter. The tRNA<sup>Ser</sup>-CCR5sh cassette was amplified from p1133-2 (Haushalter K, unpublished data) with the following primers (forward: 5'-ATG CGC CGG CAT CGA TGA AAA TGA CTT TGC CAC GCT TAG CAT GTG ACG AGG TGG CCG AGT-3' and reverse: ATG CGG CGC CAT TTA AAT AAA AAA GTG TCA AGT CCA ATC TAT GAT CTC TTG AAT CAT AGA-3') flanked by *NaeI* and *SwaI* sites on the 5' end and *NarI* and *Clal* sites on the 3' end. The *NaeI*-*NarI* and *SwaI*-*Clal* fragments were subcloned into pcDNA3-U1-MCM7-U1t plasmids<sup>21</sup> containing three triple combinations of small anti-HIV RNAs with the *Clal* and *SwaI* sites ~70 bases upstream of the 3' splice signal to generate clones in both forward and reverse orientations, respectively [pcDNA3-U1-MCM7-CCR5sh-U1t]. U1-MCM7-CCR5sh-U1t fragments were excised from pcDNA3-U1-MCM7-CCR5sh-U1t plasmids with *NruI* and *NotI* digestion and subcloned into pHIV7-GFP-P2A-MGMT<sup>P140K</sup> vector to generate SGLVs.

SGLV7 was generated by inserting the U6-U16TAR fragment into SGLV4. The U6-U16TAR fragment was generated from linearization of the pTZ/U6-U16TAR plasmid<sup>31</sup> by *BamHI* digestion, followed by fill-in of the overhang by Klenow fragment to create a blunt end, and then digested with *SphI*. This fragment was then ligated into SGLV4 that had been similarly treated except with the initial linearization with *NotI* enzyme.

**Cell culture and adult HSPC isolation.** HEK 293, HEK 293T, and HT1080 cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in complete DMEM (high glucose (4.5 g/l) Dulbecco's modified Eagle's media (DMEM) supplemented with 2 mmol/l glutamine and 10% fetal bovine serum (FBS)). CEM T lymphocytes were maintained in Roswell Park Memorial Institute 1640 media supplemented with 10% FBS.

U373-MAGI-CCR5E cells were obtained through the National Institutes of Health (NIH) AIDS Reagent Program<sup>27</sup> and maintained in complete DMEM, supplemented with 0.2 mg/ml of G418, 0.1 mg/ml of hygromycin B, and 1.0 µg/ml of puromycin.

Adult HSPCs were isolated from granulocyte colony stimulating factor (G-CSF) mobilized peripheral blood (purchased from Progenitor Cell Therapy, Mountain View, CA) from health donors under consent and in accordance to the institutional review board approval using

previously described methods.<sup>32</sup> Briefly, the washed concentrated mobilized peripheral blood were labeled with CliniMACS CD34 microbead (Miltenyi Biotec, Auburn, CA) and selected with the CliniMACS Cell Separation System (Miltenyi Biotec, Auburn, CA). The purity of the selected HSPCs was above 95% by fluorescence-activated cell sorting analysis. HSPCs were cryopreserved using a controlled rate freezer and stored in liquid nitrogen until use.

**Lentiviral vector production.** Lentiviral vectors were packaged in HEK 293T cells by calcium phosphate precipitation as previously described.<sup>33</sup> Briefly, 15 µg of transfer plasmid was cotransfected with helper plasmids (15 µg of pCMV-Pol/Gag, 5 µg of pCMV-Rev, and 5 µg of pCMV-VSVG) into HEK 293T cells with 90–95% confluency per 10-cm dish. Viral supernatant was harvested 48 hours posttransfection, concentrated by ultracentrifugation, and stored at –80 °C until use. Viral titers were determined by transduction of HT1080 cells and analyzed for EGFP expression with fluorescence-activated cell sorting analysis.

**Vector transduction.** CEM T lymphocytes were transduced with lentiviral vectors at a multiplicity of infection of 0.5 or 2.5 in the presence of 4 µg/ml of polybrene (EMD Millipore, Billerica, MA) enhanced by centrifugation. Cells with around 30% EGFP expression were expanded and sorted to purity for further experiments.

For transduced HSPCs used for *in vitro* CFU assay setup and macrophage differentiation in HIV challenge, HSPCs were thawed and prestimulated in StemSpan-SFT6 media (StemSpan (Stem Cell Technologies, Vancouver, British Columbia, Canada) supplemented with 100 ng/ml of stem cell factor (Gibco, Grand Island, NY), 100 ng/ml of Flt-3 ligand (CellGenix, Freiburg, Germany), 10 ng/ml of thrombopoietin (CellGenix), 50 ng/ml interleukin-6 (Life Technologies, Carlsbad, CA)) for 48 hours prior to transduction. Lentiviral vectors adjusted to a multiplicity of infection of 20 were added to 6.4 × 10<sup>4</sup> prestimulated HSPCs in 250 µl StemSpan-SFT6 media in the presence of 20 µg/ml of rapamycin (Sigma-Aldrich, St. Louis, MO) on RetroNectin (Takara, Mountain View, CA)-coated 96-well plate. After 24 hours of incubation, the lentiviral vector and rapamycin were removed, and HSPCs were cultured in 1.3-ml StemSpan-SFT6 media supplemented with 0.75 µmol/l SR1 (Cellagen Technologies, San Diego, CA) for 5 days prior to sorting. HSPCs were sorted on CD34 marker, in addition to EGFP expression for the transduced population. Only CD34<sup>+</sup> (untransduced) or CD34<sup>+</sup>/EGFP<sup>+</sup> (transduced) cells were used for subsequent colony forming assay setup and macrophage differentiation for HIV challenge experiments.

For transduced HSPCs used for mice transplantation, HSPCs were prestimulated overnight in StemSpan-SFT6 media and then transduced with FGLV at a multiplicity of infection of 10 at 1 × 10<sup>6</sup> cell/ml on RetroNectin (Takara, Otsu-Shiga, Japan)-coated non-tissue culture T-75 flask (5 µg protein/cm<sup>2</sup>, 1.3–2.6 × 10<sup>5</sup> viable cells/cm<sup>2</sup>) for 24 hours in the presence of 20 µg/ml of rapamycin. Lentivirus and rapamycin were removed, and cells were washed once before being resuspended for transplantation in injection saline solution (APP Pharmaceuticals, Lake Zurich, IL)

**Generation of adult HSPC-derived macrophages.** Sorted HSPCs were cultured in Iscove's modified Dulbecco's media with 20% FBS supplemented with 2 mmol/l of glutamine, 25 ng/ml of stem cell factor (Gibco), 30 ng/ml of Flt3-L (CellGenix), 30 ng/ml of interleukin-3 (Gibco), and 30 ng/ml of macrophage colony stimulating factor (PeproTech, Rocky Hill, NJ) for 10 days for guided differentiation to monocytes and were then switched to DMEM with 10% FBS supplemented with 2 mmol/l of glutamine, 10 ng/ml of granulocyte macrophage colony stimulating factor (Leukine, Sanofi US, Bridgewater, NJ), and 10 ng/ml of macrophage colony stimulating factor (PeproTech) for 5 days for activation into macrophages. Adherent macrophage cells were collected for HIV challenge experiments. The purity of cells was typically greater than 90% CD14<sup>+</sup> based on fluorescence-activated cell sorting analysis.

**Flow cytometric assay for CCR5 knockdown studies in U373-MAGI-CCR5E cells.** About  $1 \times 10^5$  U373-MAGI-CCR5E cells were seeded per well in a 24-well dish 1 day prior to transfection. An equal ratio of pHIV7-EGFP to tRNA<sup>ser</sup> construct in pBluescript with 400 ng of total plasmid DNA was transiently transfected with Lipofectamine 2000 (Life Technologies) following manufacturer's instructions. The EGFP marker from the cotransfected pHIV7-EGFP plasmid serves as an internal control for transfection efficiency. Cells were detached 72 hours later and stained with CCR5-APC antibody (clone 2D7, BD Pharmingen, San Jose, CA) to estimate CCR5 knockdown by flow cytometry. Data were collected on Gallios flow cytometer (Beckman Coulter, Brea, CA) and analyzed by FCS express version 4 software (De Novo software, Los Angeles, CA).

**Real-time quantitative reverse transcription PCR for CCR5 knockdown studies in macrophages differentiated from adult HSPCs.** Total RNA from HSPC-derived macrophages was extracted with STAT-60 reagent (Tel-Test, Friendswood, TX) according to manufacturer's protocol and then resuspended in nuclease-free water. Residual DNA was digested with Ambion TURBO DNase (Life Technologies) with 2  $\mu$ g of total RNA in a 15- $\mu$ l reaction, in accordance with manufacturer's instructions. Complementary DNA was then synthesized with 1  $\mu$ g of DNase-treated RNA with 100 ng of random primers (Invitrogen, Carlsbad, CA) and Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a 27- $\mu$ l reaction according to manufacturer's instructions. Real-time PCR was carried out with CFX96 real-time detection system with 10 ng of complementary DNA, 0.4  $\mu$ mol/l of each gene-specific (CCR5 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) primers with iQ SYBR green supermix (Bio-Rad, Hercules, CA) in a final volume of 25  $\mu$ l. The PCR conditions were 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 20 seconds and at 62 °C for 1 minute. A standard curve with known serial dilutions of total RNA input was utilized to calculate the ratio between CCR5 and GAPDH to estimate the percentage of CCR5 downregulation.

Primer sequences for PCR were as follows:

CCR5-F: 5'-TTC ATT ACA CCT GCA GCT CTC-3';  
 CCR5-R: 5'-CCT GTT AGA GCT ACT GCA ATT AT-3';  
 GAPDH-F: 5'-CGC TCT CTG CTC CTC CTG TT-3';  
 GAPDH-R: 5'-CCA TGG TGT CTG AGC GAT GT-3'.

**Northern blotting for small RNA expression analysis.** Approximately 2  $\mu$ g of pcDNA3-U1-S1/S2M/S3B-U1t with tRNA<sup>ser</sup>-CCR5sh cassette in either orientation was transfected into HEK 293 cells with 90–95% confluency per well in six-well dish with Lipofectamine 2000 (Life Technologies) according to manufacturer's protocol. Total RNA was extracted 48 hours later with STAT-60 (Tel-Test) according to manufacturer's protocol. For each construct, 10  $\mu$ g of total RNA was loaded onto 8% polyacrylamide gel with 8 mol/l urea and then electroblotted onto a Hybond-N nylon membrane (Amersham, Arlington Heights, IL) and hybridized with P<sup>32</sup>-labeled DNA probe specific for small RNA. Small nuclear U2A RNA serves as internal control.

Total RNA from sorted CEM T lymphocytes was isolated similarly, and northern blotting was performed as described above, with the exception of 20  $\mu$ g of total RNA used as input.

The DNA probes for each of small RNAs are as follows:

S1: 5'-GCG GAG ACA GCG ACG AAG AGC-3';  
 S2M: 5'-GCC TGT GCC TCT TCA GCT ACC-3';  
 S3B: 5'-CAT CTC CTA TGG CAG GAA GAA-3';  
 CCR5sh: 5'-AAA GTG TCA AGT CCA ATC TAT GA-3';  
 U5RZ: 5'-GAG TGC TTT TCG AAA ACT CAT CAG AA-3';  
 TAR: 5'-CCA GAG AGC TCC CAG GCT CAG-3';  
 CCR5RZ: 5' GTG TCA AGT TTC GTC CAC ACG GAC TCA TCA GCA ATC TA-3';  
 U2A: 5'-AGA ACA GAT ACT ACA CTT GA-3'.

**HIV challenge and intracellular HIV staining to monitor viral replication.** About  $1 \times 10^5$  HSPC-derived macrophages seeded in 48-well plate were infected in triplicate with HIV-1 Bal virus at a multiplicity of infection of 0.01 in 500  $\mu$ l volume. After overnight incubation, the HIV virus was removed, and the cells were cultured in 500  $\mu$ l DMEM with 10% FBS supplemented with 2 mmol/l of glutamine, 10 ng/ml of granulocyte macrophage colony stimulating factor, and 10 ng/ml of macrophage colony stimulating factor.

Viral replication was analyzed by intracellular staining of HIV core proteins at indicated time points. Cells were detached with Accutase solution (Sigma-Aldrich), viability was estimated by LIVE/DEAD fixable aqua dead cell stain kit (Sigma-Aldrich), and then fixed and permeabilized with intracellular fixation and permeabilization buffer set (eBioscience, San Diego, CA), before staining with KC57-RD antibody (KC57-RD1 antibody, clone FH190-1-1; Beckman Coulter) that recognizes HIV-1 core proteins, and finally analyzed by flow cytometry. Data were collected on Gallios flow cytometer and analyzed by FCS express version 4 software.

**In vitro CFU assay for adult HSPCs.** A total of 500 sorted HSPCs were plated in triplicate in MethoCult H4435-enriched methylcellulose media (Stem Cell Technologies) according to manufacturer's protocol. Cells were cultured for 12 to 13 days before colony scoring under inverted microscope.

**Fc/IL-7 protein production.** Fc/IL-7 was cloned into an OptiVect-TOPO (Invitrogen) vector, and protein was produced from a cloned transfected DG44 CHO cell line as per the methods of Lo *et al.*<sup>34</sup>

**Humanized NSG mouse model.** NOD.Cg-Prkdc<sup>scid</sup> IL2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at the City of Hope Animal Resources Center according to the protocols approved by the Institutional Animal Care and Use Committee of the City of Hope. Adult (8–10 weeks old) NSG mice were irradiated at 270 cGy for 24 hours prior to transplantation. Each animal was transplanted with  $1 \times 10^6$  HSPCs following transduction via intravenous injection. To enhance T lymphopoiesis, 20  $\mu$ g of Fc/IL-7 protein was administered per animal intravenously weekly for 11 weeks.

**In vivo O<sup>6</sup>-BG and BCNU selection for enrichment of gene-modified cells.** O<sup>6</sup>-BG (Sigma-Aldrich) was prepared in 4% dimethylsulfoxide, 30% polyethylene glycol 400 (Sigma-Aldrich) and 66% injection saline solution. BCNU (injectable carmustine) was purchased from Bristol-Myers Squibb (New York, NY) and stock solution was reconstituted in supplied absolute ethanol at 100 mg/3 ml, as per manufacturer's instructions, and then diluted in injection saline solution before administration.

Drug selection was performed either at the 7<sup>th</sup> and 8<sup>th</sup> weeks (2 $\times$  treatment cohort,  $N = 12$  mice) or at the 7<sup>th</sup>, 8<sup>th</sup>, and 9<sup>th</sup> weeks (3 $\times$  treatment cohort,  $N = 12$  mice) posttransplantation. Control cohort ( $N = 12$  mice) received saline injection. Animals in treatment cohorts received 20 mg/kg of O<sup>6</sup>-BG followed by 5 mg/kg of BCNU 1.5 hour later via intraperitoneal injection for each round of drug selection.

**Flow cytometric analysis of engraftment and gene modification frequency.** Mice were necropsied 11 weeks after transplantation for analysis of engraftment and enrichment of gene-modified cells. Single cell suspensions of bone marrow (femurs) and spleen were prepared by mechanical dissociation, and red cells were lysed using ACK lysis buffer (Sigma-Aldrich). All cell suspensions were pretreated with human immunoglobulin (GammaGard, Baxter Healthcare, Deerfield, IL) for 30 minutes to block nonspecific antibody staining. Splenocytes were stained with a human pan-leukocyte antibody to CD45-PC5 (BioLegend, San Diego, CA), and lineage-specific anti-human antibodies, CD3-ECD, CD4-APC, and CD14-APC-Alexa-750 (Invitrogen) for 20 minutes and washed two times with 1 ml of phosphate-buffered saline containing 0.1% bovine serum albumin (Sigma-Aldrich). Bone marrow cells were stained with antibodies to human CD45-PC5 (Beckman Coulter) for 20 minutes and

washed two times with 1 ml of phosphate-buffered saline containing 0.1% bovine serum albumin. To establish analytical gates and background staining, bone marrow and spleen samples from two to three untransplanted mice were stained with the same antibody panel. Data were collected on Gallios flow cytometer and analyzed by FCS express version 4 software.

**Statistical analysis.** *In vitro* CFU data were analyzed with statistical software Prism version 6.01 (GraphPad Software, La Jolla, CA), using one-way analysis of variance followed by Bonferroni's comparison test. Values of  $P \leq 0.05$  were considered statically significant compared with untransduced control. The average and standard deviation were derived from two to three independent donors as indicated.

*In vivo* drug selection data were also analyzed with Prism software using two-way analysis of variance followed by two-tailed *t*-test. Values of  $P \leq 0.05$  were considered statistically significant compared with control animals. The average and standard deviation were derived from cohorts of 12 animals per group.

## SUPPLEMENTARY MATERIAL

**Figure S1.** Potent antiviral activity of combinatorial RNA vectors in stably expressing CEM T lymphocytes.

### Materials and Methods

## ACKNOWLEDGMENTS

The following reagent was obtained through the National Institutes of Health (NIH) AIDS Reagent Program, Division of AIDS, NIAID, NIH: U373-MAGI-CCR5E from Michael Emerman.

The authors declare no competing financial interests. The authors thank Lijing Li and Anitha Rao for optimization of seeding density for *in vitro* CFU assay. They thank Lucy Brown and Alex Spalla in the analytical flow cytometry core for cell sorting. This work was funded by CIRM TR2-01771 to D.L.D. and J.J.R. This publication is the sole responsibility of the authors and does not necessarily represent the views of the CIRM or the state of California.

## REFERENCES

- World Health Organization (October 2013). HIV/AIDS Fac Sheet N°360.
- Calza, L (2012). Renal toxicity associated with antiretroviral therapy. *HIV Clin Trials* **13**: 189–211.
- Jones, M and Núñez, M (2012). Liver toxicity of antiretroviral drugs. *Semin Liver Dis* **32**: 167–176.
- Lipshultz, SE, Mas, CM, Henkel, JM, Franco, VI, Fisher, SD and Miller, TL (2012). HAART to heart: highly active antiretroviral therapy and the risk of cardiovascular disease in HIV-infected or exposed children and adults. *Expert Rev Anti Infect Ther* **10**: 661–674.
- Domingo, P, Estrada, V, López-Aldeguer, J, Villaroya, F and Martínez, E (2012). Fat redistribution syndromes associated with HIV-1 infection and combination antiretroviral therapy. *AIDS Rev* **14**: 112–123.
- Hester, EK (2012). HIV medications: an update and review of metabolic complications. *Nutr Clin Pract* **27**: 51–64.
- Bauer, G and Anderson, JS (2014). Stem cell transplantation in the context of HIV—how can we cure HIV infection? *Expert Rev Clin Immunol* **10**: 107–116.
- Stan, R and Zaia, JA (2014). *Practical considerations in gene therapy for HIV cure*. Current HIV/AIDS reports.
- Younan, P, Kowalski, J and Kiem, HP (2014). Genetically modified hematopoietic stem cell transplantation for HIV-1-infected patients: can we achieve a cure? *Mol Ther* **22**: 257–264.
- Boutimah, F, Eekels, JJ, Liu, YP and Berkhout, B (2013). Antiviral strategies combining antiretroviral drugs with RNAi-mediated attack on HIV-1 and cellular co-factors. *Antiviral Res* **98**: 121–129.
- ter Brake, O, 't Hooft, K, Liu, YP, Centlivre, M, von Eije, KJ and Berkhout, B (2008). Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. *Mol Ther* **16**: 557–564.
- Ringpis, GE, Shimizu, S, Arokium, H, Camba-Colón, J, Carroll, MV, Cortado, R *et al.* (2012). Engineering HIV-1-resistant T-cells from short-hairpin RNA-expressing hematopoietic stem/progenitor cells in humanized BLT mice. *PLoS One* **7**: e33492.

- Liu, YP, Haasnoot, J, ter Brake, O, Berkhout, B and Konstantinova, P (2008). Inhibition of HIV-1 by multiple siRNAs expressed from a single microRNA polycistron. *Nucleic Acids Res* **36**: 2811–2824.
- Aagaard, LA, Zhang, J, von Eije, KJ, Li, H, Saetrom, P, Amarzguoui, M *et al.* (2008). Engineering and optimization of the miR-106b cluster for ectopic expression of multiplexed anti-HIV RNAs. *Gene Ther* **15**: 1536–1549.
- Saayman, S, Arbuthnot, P and Weinberg, MS (2010). Deriving four functional anti-HIV siRNAs from a single Pol III-generated transcript comprising two adjacent long hairpin RNA precursors. *Nucleic Acids Res* **38**: 6652–6663.
- Li, MJ, Bauer, G, Michienzi, A, Yee, JK, Lee, NS, Kim, J *et al.* (2003). Inhibition of HIV-1 infection by lentiviral vectors expressing Pol III-promoted anti-HIV RNAs. *Mol Ther* **8**: 196–206.
- Walker, JE, Chen, RX, McGee, J, Nacey, C, Pollard, RB, Abedi, M *et al.* (2012). Generation of an HIV-1-resistant immune system with CD34(+) hematopoietic stem cells transduced with a triple-combination anti-HIV lentiviral vector. *J Virol* **86**: 5719–5729.
- Li, MJ, Kim, J, Li, S, Zaia, J, Yee, JK, Anderson, J *et al.* (2005). Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. *Mol Ther* **12**: 900–909.
- DiGiusto, DL, Krishnan, A, Li, L, Li, H, Li, S, Rao, A *et al.* (2010). RNA-based gene therapy for HIV with lentiviral vector-modified CD34(+) cells in patients undergoing transplantation for AIDS-related lymphoma. *Sci Transl Med* **2**: 36ra43.
- DiGiusto, DL, Stan, R, Krishnan, A, Li, H, Rossi, JJ and Zaia, JA (2013). Development of hematopoietic stem cell based gene therapy for HIV-1 infection: considerations for proof of concept studies and translation to standard medical practice. *Viruses* **5**: 2898–2919.
- Chung, J, Zhang, J, Li, H, Ouellet, DL, DiGiusto, DL and Rossi, JJ (2012). Endogenous MCM7 microRNA cluster as a novel platform to multiplex small interfering and nucleolar RNAs for combinational HIV-1 gene therapy. *Hum Gene Ther* **23**: 1200–1208.
- Gori, JL, Beard, BC, Ironside, C, Karponi, G and Kiem, HP (2012). *In vivo* selection of autologous MGMT gene-modified cells following reduced-intensity conditioning with BCNU and temozolomide in the dog model. *Cancer Gene Ther* **19**: 523–529.
- Beard, BC, Trobridge, GD, Ironside, C, McCune, JS, Adair, JE and Kiem, HP (2010). Efficient and stable MGMT-mediated selection of long-term repopulating stem cells in nonhuman primates. *J Clin Invest* **120**: 2345–2354.
- Trobridge, GD, Wu, RA, Beard, BC, Chiu, SY, Muñoz, NM, von Laer, D *et al.* (2009). Protection of stem cell-derived lymphocytes in a primate AIDS gene therapy model after *in vivo* selection. *PLoS One* **4**: e7693.
- Yam, PY, Li, S, Wu, J, Hu, J, Zaia, JA and Yee, JK (2002). Design of HIV vectors for efficient gene delivery into human hematopoietic cells. *Mol Ther* **5**: 479–484.
- Scherer, LJ, Frank, R and Rossi, JJ (2007). Optimization and characterization of tRNA-shRNA expression constructs. *Nucleic Acids Res* **35**: 2620–2628.
- Vodicka, MA, Goh, WC, Wu, LI, Rogel, ME, Bartz, SR, Schweickart, VL *et al.* (1997). Indicator cell lines for detection of primary strains of human and simian immunodeficiency viruses. *Virology* **233**: 193–198.
- Chassagne, J, Verrelle, P, Dionet, C, Clavel, F, Barre-Sinoussi, F, Chermann, JC *et al.* (1986). A monoclonal antibody against LAV gag precursor: use for viral protein analysis and antigenic expression in infected cells. *J Immunol* **136**: 1442–1445.
- Ouellet, DL, Plante, I, Landry, P, Barat, C, Janelle, ME, Flamand, L *et al.* (2008). Identification of functional microRNAs released through asymmetrical processing of HIV-1 TAR element. *Nucleic Acids Res* **36**: 2353–2365.
- Younan, PM, Polacino, P, Kowalski, JP, Peterson, CW, Maurice, NJ, Williams, NP *et al.* (2013). Positive selection of mC46-expressing CD4+ T cells and maintenance of virus specific immunity in a primate AIDS model. *Blood* **122**: 179–187.
- Michienzi, A, Li, S, Zaia, JA and Rossi, JJ (2002). A nucleolar TAR decoy inhibitor of HIV-1 replication. *Proc Natl Acad Sci USA* **99**: 14047–14052.
- Tran, CA, Torres-Coronado, M, Gardner, A, Gu, A, Vu, H, Rao, A *et al.* (2012). Optimized processing of growth factor mobilized peripheral blood CD34+ products by counterflow centrifugal elutriation. *Stem Cells Transl Med* **1**: 422–429.
- Li, MJ and Rossi, JJ (2005). Lentiviral vector delivery of recombinant small interfering RNA expression cassettes. *Methods Enzymol* **392**: 218–226.
- Lo, KM, Sudo, Y, Chen, J, Li, Y, Lan, Y, Kong, SM *et al.* (1998). High level expression and secretion of Fc-X fusion proteins in mammalian cells. *Protein Eng* **11**: 495–500.



This work is licensed under a Creative Commons Attribution-NonCommercial-No Derivative Works 3.0 License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0/>