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Reducing the Genotoxic Potential of Retroviral Vectors

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Abstract

The recent development of leukemia in gene therapy patients with X-linked severe combined immunodeficiency disease due to retroviral vector insertional mutagenesis has prompted reassessment of the genotoxic potential of integrating vector systems. In this chapter, various strategies are described to reduce the associated risks of retroviral genomic integration. These include deletion of strong transcriptional enhancer-promoter elements in the retroviral long terminal repeats, flanking the retroviral transcriptional unit with enhancer blocking sequences and designing vectors with improved RNA 3' end processing. Protocols are provided to evaluate the relative biosafety of the modified vectors based on their ability to immortalize hematopoietic progenitor cells and propensity to trigger clonal hematopoiesis or leukemogenesis following hematopoietic stem cell transplantation.

Keywords

Gammaretroviral vectors; lentiviral vectors; insertional mutagenesis; genotoxicity; self-inactivating vectors; enhancer blocking sequences; posttranscriptional regulatory elements; RNA 3' end processing.

1. Introduction

Until 2003, retroviral vectors had been considered a relatively safe means for gene delivery to human hematopoietic stem cells (HSCs), with no serious adverse events related to the vector systems reported in at least 40 gene marking and gene therapy trials involving more than 200 patients (1,2). A caveat of these observations is that most of the early clinical studies were characterized by very low efficiencies of gene transfer. This proviso notwithstanding, no progression to clonal hematopoiesis or leukemia had been observed to this point in preclinical investigations involving nonhuman primates or dogs followed for up to 7 years after receiving retrovirally-transduced HSCs, including instances where significant levels of gene transfer had been achieved (3). However, the subsequent emergence of leukemia in 3 gene therapy patients with X-linked severe combined immunodeficiency disease (X-SCID) due to insertional activation of the *LMO2* proto-oncogene has prompted reevaluation of the safety profile of retroviral-mediated gene delivery (4-7). Other recent preclinical and clinical follow-up studies have provided additional evidence of the potentially dangerous consequences of retroviral chromosomal insertion events (8-16) .

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While retroviral DNA integration is not site-specific, genome-wide analyses of integration sites have revealed that murine leukemia virus (MLV)-based gammaretroviral vectors and human immunodeficiency virus type 1 (HIV-1)-based lentiviral vectors do not integrate at random throughout the genome. Rather, they preferentially insert their cargos into open chromatin regions in close proximity to or within genes (6,17,18). Whereas MLV exhibits a strong bias to integrate within a 5-kb window upstream or downstream of transcription initiation sites, HIV-1 tends to target the transcriptional unit itself. It is clear therefore that gene activation or disruption as a side effect of the retroviral integration process is greater than what would be predicted based on the assumption of a random distribution of sites throughout the genome. In this context, it is important to emphasize that MLV belongs to the slow transforming group of retroviridae (aka oncoretroviruses) and that its ability to induce hematopoietic tumor formation in mice is precisely because of its capacity to activate cellular proto-oncogenes – ergo the origin of the term 'retroviral insertional mutagenesis' (19-21). Nonetheless, although MLV is known to transform primate cells when allowed to replicate (22), in view of a multistep mechanism of malignant transformation in humans (23,24), the risk of leukemogenesis from replication-defective MLV-based gammaretroviral vectors (16) was believed to be low. Similarly, clonal dominance – the predominant occurrence of a few retroviral vector-marked HSC-derived hematopoietic clones following bone marrow transplantation – had been thought to be a natural property of hematopoiesis (25-27). However, insertion site analysis in recent preclinical and clinical gene transfer studies has indicated that retroviral integration may influence this process by activating genes involved in growth control – such as the *Evi1* oncogene (8,13,14,28) – leading in some cases to selective nonmalignant clonal outgrowth (9,15). Consequently, the combinatorial effects of multiple gammaretroviral vector insertions within a cell could lead to oncogenic conversion, especially if the transgene product itself confers a survival or proliferative advantage to the target cell population (8,10,29,30), as may be the case with the common gamma chain (γ_c) cytokine receptor gene in X-SCID (31,32). Though most safety concerns associated with insertional mutagenesis have to do with gammaretroviral vector-induced transcriptional activation of genes, it is not unreasonable to assume that haploinsufficiency of tumor suppressor genes due to lentiviral vector-mediated gene disruption could predispose toward malignancy (12,33-37). In any case, obtaining a low copy number of vector integrants per cell is clearly desirable (10,35).

Besides reducing the frequency of multiple integration events per cell, several safety modifications of the retroviral vector design can be envisioned (for reviews, see (6,7,38,39)). MLV transcriptional activation of cellular gene expression results from the strong enhancer-promoter elements in the retroviral long terminal repeats (LTRs), with enhancer-mediated gene activation being the most frequent mechanism (19-21). Therefore, deletion of the LTR enhancer elements would be presumed to provide some modicum of improvement in terms of safety. Retroviral vectors having this design are referred to as 'self-inactivating' (SIN) vectors (40-43). With the exception of our HHAM vector platform (26,27,41), the titers of the initial versions of most SIN gammaretroviral vectors were too low to be of utility for HSC gene transfer. However, recent advances have resulted in SIN gammaretroviral vector titers approaching those of conventional LTR vectors (44-46).

In the absence of the LTR enhancer-promoter elements, SIN retroviral vectors utilize an internal enhancer-promoter to drive transgene transcription. Therefore, although effectively reducing the copy number of enhancer elements per vector by a factor of 2, there is still a potential for the internal enhancer in these vectors to activate cellular gene expression. A strategy being implemented to prevent this involves flanking the vector sequences with insulators or enhancer blocking sequences (38,47,48). We and others have introduced a 1.2-kb fragment containing a monomer of the chicken β-globin 5' hypersensitive site 4 (5'HS4) insulator, which functions as a chromatin domain boundary, into the U3 region of retroviral LTRs (49-54). However, the titers of these 5'HS4 insulator-containing 'double-copy' vectors

(55,56) are generally reduced. Moreover, superior protection in cell culture transfection and *Drosophila* transgenic experiments is obtained when the transgene is flanked by two tandem copies of the 1.2-kb 5'HS4 insulator fragment (57). In extensive work by the Felsenfeld group, a GC-rich core element of the 5'HS4 insulator has been mapped to a 5' 250-bp fragment (58), two tandem copies of which were shown to provide complete insulator function (59). Therefore, more recent approaches have involved insertion of a dimer of the smaller 250-bp 5'HS4 insulator core element into the LTR U3 regions (45,60). Because almost all of the enhancer blocking activity of the 1.2-kb 5'HS4 insulator fragment can be conferred by a 42-bp sequence that is bound by CTCF (CCCTC-binding factor), a highly conserved and ubiquitous DNA-binding protein implicated in both transcriptional silencing and activation (59,61), it might be possible to further minimize the 5'HS4 insulator sequences without compromising any improvements gained in safety with the larger fragments.

It is worth noting that many of the existing SIN vectors (including SIN lentiviral vectors) are not necessarily completely transcriptionally disabled, either because the LTR is not fully inactivated (40,45,62,63) or because of residual transcriptional regulatory elements within the 5' untranslated region of viral RNA that may contribute to the activation of cryptic promoters (64,65). Moreover, transcription termination is especially leaky in the SIN vector format (66). In addition to promoting mRNA nuclear export, the eukaryotic splicing process results in enhanced 3' end formation and polyadenylation (67). Therefore, engineering introns within the vector may also reduce 3' RNA readthrough (44,45,66,68). An alternative approach is the inclusion of *cis*-acting RNA transport elements such as the woodchuck hepatitis virus posttranscriptional regulatory element, which also augments RNA 3' end processing and polyadenylation (68-70). In this regard, a safety-modified version of this posttranscriptional regulatory element devoid of potentially oncogenic promoter sequences and open reading frames that were present in the original version has recently been described (71). A prototypical SIN retroviral vector incorporating all of the features discussed above is illustrated in Fig. 1.

This chapter provides protocols to assess the relative genotoxicity of different retroviral vector designs and to systematically evaluate whether the proposed modifications enhance vector safety.

2. Materials

2.1. Vector Production

2.1.1 Transient Transfection

1. 293T/17 (293T) human embryonic kidney cell line (American Type Culture Collection, Manassas, VA; cat. no. CRL-11268) (*see* **Note 1**).
2. 293T cell growth medium: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4.5 g/L glucose, 2 m*M* L-glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). Store at 4°C and warm up to 37°C before use. This growth medium is also used to culture NIH3T3 cells.
3. Gammaretroviral or lentiviral vector plasmid DNA containing the gene of interest (*see* **Note 2**).
4. Gammaretroviral (e.g., pEQPAM3-E; (74)) or lentiviral (e.g., pCMVΔR8.91; (75)) packaging construct plasmid DNA.
5. Vesicular stomatitis virus G (VSV-G) glycoprotein (e.g., pMD.G; (76,77)) or MLV ecotropic envelope plasmid DNA (e.g., pCAG4-Eco; (78,79)).

6. 2.5 M $CaCl_2$: Dissolve 183.7 g $CaCl_2$ dihydrate (tissue culture grade) in deionized, distilled water. Bring the volume up to 500 mL and filter-sterilize using a 0.22-μm nitrocellulose filter. Stable at −20°C.
7. 2× *N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid) (HEPES)-buffered saline (2× HBS): 50 m*M* HEPES (Sigma-Aldrich Corp., St. Louis, MO; cat. no. H4034), 280 m*M* NaCl, 1.5 m*M* Na_2PHO_4. Titrate to pH 7.05 with 5 *N* NaOH. Filter sterilize using a 0.22-μm nitrocellulose filter. Store as single use aliquots at −20°C.
8. 1 M HEPES in 0.85% NaCl (Cambrex Bio Science Walkersville, Inc., Walkersville, MD; cat. no. 17−737F). Store at 4°C.

2.1.2 Collection and Concentration of Vector Particles

1. Low protein binding Durapore (PVDF) 0.45-μm filter unit (Millipore Corp., Bedford, MA).
2. Stericup 150 mL 0.45 μm filter unit (Millipore Corp.).

2.1.3 Titration of Vector Stocks

1. NIH3T3 murine fibroblasts (ATCC cat. no. CRL-1658).
2. Polybrene stock solution (Sigma-Aldrich Corp.; hexadimethrine bromide, cat no. H9268). Prepare stock of 6 mg/mL (1000×) in sterile deionized, distilled water; aliquot and store at −20°C.
3. Geneticin (Sigma-Aldrich Corp.; G418 disulfate salt, cat. no. A1720). Prepare stock of 40 mg/mL in sterile deionized, distilled water; aliquot and store at −20°C.

2.2. Insertional Mutagenesis Assays

2.2.1. Immortalization of Hematopoietic Progenitor Cells

1. Female C57BL/6 mice (6- to 8-wk old; The Jackson Laboratory, Bar Harbor, ME; cat. no. 000664) used as bone marrow donors. All procedures involving mice must follow the guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and be approved by an Institutional Animal Care and Use Committee.
2. 5-fluorouracil (5-FU; Sigma-Aldrich Corp., cat. no. F6627). Store the stock at room temperature, avoiding light. Prepare a fresh working solution of 15 mg/mL in phosphate-buffered saline (PBS) immediately before use.
3. Erythrocyte lysing solution: 154 m*M* NH_4Cl, 10 m*M* $NaHCO_3$, and 0.082 m*M* sodium ethylenediaminetetraacetic acid (EDTA), pH 7.3. Commercial lysing solutions are also available. Store at room temperature.
4. Bone marrow progenitor cell growth medium: Iscove's modified Dulbecco's medium (IMDM) supplemented with 4.5 g/L glucose, 2 m*M* L-glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin, 15% heat-inactivated FBS, 100 ng/mL murine stem cell factor (SCF), 30 ng/mL murine interleukin-3 (IL-3), and 10 ng/mL murine IL-6. Store at 4°C and warm up to 37°C before use.
5. Recombinant fibronectin fragment (RetroNectin; Takara Mirus Bio, Madison, WI).
6. Polybrene stock solution (1000×; 8 mg/mL) prepared as described in **Subheading 2.1.3**. Aliquot and store at −20°C.

2.2.2. Hematopoietic Clonal Evolution and Leukemogenesis Assays

1. Female C57BL/6 mice (6- to 8-wk old; as listed in **Subheading 2.2.1**) used as bone marrow donors and recipients.
2. Small animal gamma irradiator.
3. Hematology analyzer equipped with a veterinarian software package.
4. Flow cytometer.

3. Methods

3.1. Vector Production

293T (293 human embryonic kidney cells expressing simian virus 40 [SV40] large tumor [T] antigen) cells are highly transfectable such that transient co-transfection with current generation transfer vectors and packaging plasmids yields high-titer, replication-defective gammaretroviral and lentiviral vector particles (80). Retroviral vectors pseudotyped with the VSV-G glycoprotein have a broad host-cell range and can be utilized in the insertional mutagenesis assays described herein (76,77). However, VSV-G-pseudotyped virions are frequently associated with toxicity and transduction artifacts (81-83). Moreover, because the assays involve transduction of murine HSC and progenitor populations, the MLV ecotropic envelope can be conveniently used to pseudotype any gammaretroviral or lentiviral vectors to be evaluated (79,84,85).

3.1.1 Transient Transfection

1. Culture 293T cells in 293T/NIH3T3 cell growth medium at 37°C in a humidified atmosphere with 5% CO_2.
2. Passage cells every 3 to 4 days using a trypsin-EDTA solution to dissociate them. To trypsinize, remove the medium and rinse the cells with PBS (without Ca_2^+ and Mg_2^+). Remove the PBS and add enough trypsin-EDTA to cover the cells. Incubate the plate at room temperature until the cells round up and detach. To inactivate the trypsin, add an equal volume of DMEM medium containing 10% FBS. Collect the cells and centrifuge for 5 min at 375*g*. Resuspend cells in fresh culture medium and plate between 1:4 to 1:8.
3. Transfect 293T cells with plasmid DNAs using the calcium phosphate precipitation method (86). On the day before transfection, plate 293T cells in 7 mL 293T/NIH3T3 cell growth medium at a density of 5×10^6 cells per 100-mm plate.
4. Mix 15 μg of the transfer vector plasmid, 10 μg of the appropriate packaging plasmid and 5 μg of the MLV ecotropic envelope (or VSV-G glycoprotein) plasmid (*see* **Note 3**). Bring the volume up to 400 μL with sterile water. Add 100 μL of 2.5 *M* $CaCl_2$ and mix. Add the DNA/$CaCl_2$ solution dropwise to 500 μL of 2× HBS in a 15-mL conical tube. Use a second pipettor and a 2-mL pipet to bubble the 2× HBS as the DNA/$CaCl_2$ solution is added. Vortex immediately for 5 sec and incubate at room temperature for 20 min. Add the 1 mL DNA/calcium phosphate mixture directly to each 100-mm plate while swirling (*see* **Note 4**). Incubate the cells at 37°C overnight (16 h). Change the medium and culture for 24−48 h (this method usually results in transfection of 50−80% of the cells).

3.1.2 Collection and Concentration of Vector Particles—Collect the vector-conditioned medium 24−48 h after medium change, centrifuge at 2000*g* for 10 min to remove cellular debris and filter through a 0.45 μm pore-size filter (use a 5 mL syringe or a 150 mL

filter unit depending on the volume). Use directly for transductions or aliquot and store at −80° C (*see* **Note 5**).

Several procedures have been developed to concentrate retroviral vector particles (73). However, concentration by centrifugation is the most commonly used method. While the stability of the VSV-G glycoprotein allows concentration of vector particles by ultracentrifugation, vector particles pseudotyped with the MLV ecotropic envelope should be concentrated by the following low-speed centrifugation protocol.

1. Centrifuge the vector supernatants overnight (16 h) at 13,000*g* and 4°C.
2. Remove supernatants and, using gentle pipetting, resuspend pellets in ~100 μL of medium appropriate for the downstream application. To allow complete resuspension, vortex gently overnight at 4°C.
3. To remove cellular debris, centrifuge concentrated supernatants at 10,000*g* and 4°C for 5 min in a microcentrifuge. Remove the supernatant, aliquot and freeze at −80°C (*see* **Note 6**).

3.1.3 Titration of Vector Stocks—The NIH3T3 murine fibroblast cell line can be used to determine the titer of ecotropic gammaretroviral and lentiviral vector preparations.

1. Culture NIH3T3 cells in 293T/NIH3T3 cell growth medium at 37°C in a humidified atmosphere with 5% CO_2.

2. Plate 2.5×10^5 NIH3T3 cells in each well of a 6-well plate 4−6 h before titrating the vector supernatants.

3. In a final volume of 1 mL, prepare serial dilutions for each vector preparation (e.g., 10^0, 10^{-1}, and 10^{-2} for unconcentrated vector supernatants; 10^{-2}, 10^{-3}, and 10^{-4} for concentrated supernatants) using 293T/NIH3T3 cell growth medium. To each dilution, add 1 μL of 6 mg/mL (1000×) polybrene for a final concentration of 6 μg/mL. Remove the medium from the cells and add each of the 1 mL dilutions to the corresponding well. Incubate at 37°C for 4 h.

4. Remove the supernatants after the 4-h transduction and replace with 2 mL fresh 293T/NIH3T3 cell growth medium. Return to the CO_2 incubator and incubate at 37°C.

5a. After 48 h, determine the relative end-point vector titers (in transducing units per mL [TU/mL]) by flow cytometric analysis (i.e., if the vector contains a fluorescent reporter gene such as the green fluorescent protein [GFP]) (87). To determine the vector titer, use the following equation:

Vector titer = number of NIH3T3 cells × % of GFP^+ cells × dilution factor (*see* **Note 7**).

5b. If the vector carries a drug resistance gene (88), split cells 1:10 after 48 h and seed into 100-mm dishes in medium containing the recommended amount of the corresponding drug (e.g., 400 μg/mL geneticin if the neomycin phosphotransferase [*neo*] drug resistance gene is used). Replace medium every 4 to 5 days for 2 weeks. Fix and stain cells with 0.3% crystal violet in 70% methanol and enumerate the drug-resistant colonies. To determine the vector titer, use the following equation:

Vector titer = number of drug-resistant colonies × 10 × dilution factor.

3.2. Insertional Mutagenesis Assays

3.2.1. Immortalization of Hematopoietic Progenitor Cells—In light of the leukemias that developed in 3 X-SCID patients as a result of insertional activation of the *LMO2* oncogene

(4), it is imperative that all ensuing retroviral gene therapy protocols include stringent preclinical evaluations of vector genotoxicity. Thus, irrespective of therapeutic efficacy, any HSC-based gene transfer approach will only become clinically acceptable if there is a low risk-to-benefit ratio (5-7). Recently, a retroviral insertional mutagenesis assay involving immortalization of murine bone marrow progenitors was described (14). Interestingly, ~25% of the immortalized cell lines obtained contained vector insertions in the *Evi1* gene, where they activated expression of a truncated protein similar to what is observed in human leukemias (89). It is of relevance that retroviral vector insertions in *Evi1* have also been identified in at least 6 murine bone marrow transplant recipients (8,9), 9 rhesus macaque bone marrow transplant recipients (13), and in 2 patients in a chronic granulomatosis disease clinical trial (15).

Copeland and colleagues (14) established cell lines from 50% of murine bone marrow progenitor cell cultures when 1×10^6 cells were transduced with a gammaretroviral vector harboring intact LTRs that had a titer of 3×10^6 TU/mL. Approximately 30% of the cultures comprised 2 clones, and the average number of vector copies per cell was 5−6. However, when vector titers were reduced to 4×10^5 TU/mL, no immortalized cell lines were obtained (n = 10). In contrast, when bone marrow progenitor cells were transduced with a derivative of the vector carrying an Evi1 cDNA (encoding the short Evi1 isoform expressed in many of the cell lines as a result of insertional mutagenesis), all of the cultures gave rise to immortalized cell lines with a titer of 1×10^5 TU/mL. Subcloning of these cell lines indicated single copy integration, suggesting that inappropriate expression of *Evi1* alone is sufficient to promote the immortalization of murine hematopoietic progenitor cells under these conditions. We previously obtained similar results – single copy integration in immortalized hematopoietic progenitor cell lines – using a gammaretroviral vector carrying the *HOX11*/*TLX1* oncogene (90). Therefore, insertional mutagenesis experiments should be carried out in parallel with a vector expressing *Evi1* or *HOX11*/*TLX1* (1×10^5 TU/mL) as a positive control.

1. Dissolve 5-FU in sterile PBS (15 mg/mL) immediately before use and intravenously inject into mice (150 mg/kg body weight) using a 27-gauge needle attached to a 1-mL syringe.
2. Harvest bone marrow cells 4 days after the 5-FU injection. Flush the hind limbs with PBS containing 2% FBS using a 21-gauge needle attached to a 5-mL syringe (91, 92) (*see* **Note 8**).
3. Lyse erythrocytes by incubating total bone marrow cells in erythrocyte lysis solution for 10 min at room temperature followed by centrifugation at 375*g* for 5 min.
4. Coat 35-mm suspension culture plates with 2 μg/cm^2 recombinant fibronectin fragment (93). Transfer the nucleated cells to plates at a density of 5×10^5 cells/mL and culture for 48 h in bone marrow progenitor cell growth medium at 37°C in a humidified atmosphere containing 5% CO_2.
5. Transduce the bone marrow cells for 3 consecutive days (4 h each day) by incubation with gammaretroviral or lentiviral vector conditioned medium in the presence of 8 μg/mL polybrene supplemented with the same growth factors as used for prestimulation (94).
6. Culture the transduced cells in bone marrow progenitor cell growth medium minus murine IL-6. Passage cells every 3 days. Immortalized clones that arise will continue to propagate after 1 month of culturing (95).
7. Extract genomic DNA from the immortalized cells and subject to Southern blot analysis to determine vector copy number. Perform integration site analysis (e.g., by linear amplification-mediated polymerase chain reaction [LAM-PCR]; (5)).

3.2.2. Hematopoietic Clonal Evolution and Leukemogenesis Assays—The *in vivo* effects of insertional mutagenesis – evolution to hematopoietic clonal dominance or frank leukemia – can be examined in mice transplanted with retrovirally-transduced HSCs (8-12). For example, the latency of leukemic conversion can be reduced by using a gammaretroviral vector carrying an oncogene (11). In one study, 60% of the mice transplanted with bone marrow transduced with a Sox4 gammaretroviral vector presented with myeloid leukemias between 4 and 7 months posttransplant, whereas under similar conditions no malignancies developed in animals transplanted with bone marrow cells transduced with the vector backbone alone. Although the finding is not surprising, it further reinforces the point that caution is warranted when the transgene product in a gene therapy protocol confers a selective growth or survival advantage to the target cell population (8,10,29-32). Along these lines, we previously reported sporadic leukemia development in murine transplant recipients that received transduced HSCs carrying gammaretroviral vector-encoded cytokine genes: IL-11 (one instance out of 10 primary and 18 secondary recipients) or FLT3 ligand (20 out of 24 primary recipients) (29, 30).

1. Expose C57BL/6 recipient mice to an otherwise lethal dose of total body γ-irradiation using a gamma irradiator (1050 cGy; split dose with 3 h between doses).
2. Transplant the irradiated mice with $1–2 \times 10^6$ retrovirally transduced bone marrow cells (obtained as described in **Subheading 3.2.1.1**) injected via the tail vein in 300 μL PBS plus 2% FBS using a 27-gauge needle attached to a 1-mL syringe (84). House the transplanted mice in sterile microisolator cages on laminar flow racks. Add an antibiotic to the drinking water (e.g., 2 cc/250 mL Baytril; Bayer Corp., Shawnee Mission, KS) for 3–4 weeks as a prophylactic measure during hematopoietic recovery to prevent possible deaths due to adventitious infections.
3. At biweekly intervals after transplant, collect peripheral blood from the retroorbital venous sinus and analyze hematological parameters—such as total leukocytes, total erythrocytes, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, total platelets and mean platelet volume—using a hematology analyzer equipped with a veterinarian software package. Mouse bleeding is performed after inhalation isoflurane anesthesia and administration to the eye of one drop of a local anesthetic (e.g., 0.5% Tetracaine Ophthalmic Solution, Phoenix Pharmaceutical Inc., St. Joseph, MO). Collect blood using microhematocrit capillary tubes and place ~100 μL into 1.3 mL micro-collection tubes containing potassium EDTA (1.6 mg/mL blood; Sarstedt Inc., Newton, NC; cat. no. 41.1395.105). Cap the tube and gently mix the contents of the tube by flicking the side of the tube. Keep the blood sample at room temperature for at least 5 min prior to measurement. The sample may be analyzed up to several hours after collection.
4. If no hematopathologic changes are observed within 6 months, sacrifice the mice and isolate bone marrow cells to transplant into lethally irradiated secondary recipients ($1–2 \times 10^6$ cells per mouse). Extract genomic DNA from bone marrow and/or spleen cells and perform integration site analysis (e.g., by Southern blot analysis and LAM-PCR).
5. Collect peripheral blood from the retroorbital venous sinus of the secondary recipients at biweekly intervals after transplant and analyze hematological parameters.
6. If mice display hematopathologic changes (leukocytosis) or become moribund (showing substantial weight loss, ruffled fur and a hunched posture) (29,30), collect peripheral blood from the retroorbital venous sinus and place ~100 μL into micro-collection tubes. Centrifuge at 375*g* for 5 min and resuspend the pellet in 500 μL PBS

containing 2% FBS plus 0.1% NaN_3. Sacrifice the mice and also collect cells from bone marrow, spleen, and thymus. Lyse the erythrocytes and prepare aliquots of ~5 × 10^4 cells/50 μL in PBS containing 2% FBS plus 0.1% NaN_3 for staining individually with fluorochromeconjugated lineage-specific (Gr1, Mac1, CD19, TER119, CD3, CD4, CD8) monoclonal antibodies. Incubate for 30 min at 4°C. Wash in 2 mL PBS containing 2% FBS plus 0.1% NaN_3. Centrifuge at 375*g* for 5 min. Decant supernatant and drain. Resuspend in 300 μL PBS containing 2% FBS plus 0.1% NaN_3 and analyze by flow cytometry (*see* **Note 9**). Transplant 1 × 10^6 bone marrow cells into nonirradiated recipients to determine whether any leukemias that arise are transplantable.

7. Sacrifice any mice when they display hematopathologic changes or become moribund and all asymptomatic secondary recipients 6 months after transplantation. Extract genomic DNA from bone marrow and/or spleen cells and perform integration site analysis (e.g., by Southern blot analysis and LAM-PCR).

1. The 293T/17 cell line is a derivative of the 293T (293tsA1609neo) cell line (96). 293T is a highly transfectable derivative of the 293 human embryonic kidney cell line into which the temperature sensitive gene for SV40 large T antigen was inserted. Human 293 cells express the adenovirus serotype 5 E1A 12S and 13S gene products, which strongly transactivate transcription from expression vectors containing the human cytomegalovirus immediate early region enhancer-promoter elements (97). In addition, the SV40 large T antigen may stimulate extrachromosomal replication of plasmids containing the SV40 origin of replication during transient transfection.

2. Besides academic sources, retroviral vector backbones and packaging systems are available commercially (e.g., Clontech Laboratories, Inc., Mountain View, CA; Invitrogen Corp., Carlsbad, CA; Stratagene Corp., La Jolla, CA).

3. A so-called ‘third generation’ HIV-1 lentiviral vector system has been developed in which the *rev* gene has been deleted from the *gag-pol* packaging construct (98). Since both the expression of the *gag* and *pol* genes and lentiviral vector transcripts are dependent on *trans* complementation by a separate Rev expression construct, use of this system requires cotransfection of 4 plasmids. For further details on HIV-1 lentiviral vector designs and protocols, see refs. (72,73).

4. The vector titer depends on several factors including the vector backbone design, the size and nature of any inserted sequences as well as on the efficiency of transfection. Always use an exponentially growing culture of 293T cells for transfection (50−70% confluent) and make sure that the cells are trypsinized well during plating so that they form a uniform monolayer. Another factor that affects the transfection efficiency is the quality of the plasmid DNA used. Use a commercially available plasmid DNA purification kit (e.g., from Qiagen, Valencia, CA) to obtain highly purified endotoxin-free supercoiled plasmid DNA (traditionally obtained by purifying on two separate cesium chloride gradients). Sterilize the DNA by ethanol precipitation, resuspend the air-dried pellet in sterile deionized, distilled water, and determine its concentration and quality by spectrophotometric analysis and gel electrophoresis. Also of importance with regard to the transfection efficiency is the pH of the 2× HBS solution, which should be between 7.05 and 7.12. Once the transfection mixture has been added to the cells, a fine precipitate should develop within a few minutes.

5. Vector titers are also influenced by factors that affect the stability of the vector particles, which include the pH, temperature and freeze-and-thaw frequency (99). Avoid pH changes in vector supernatants as this could lead to significant loss of titer. pH changes can be prevented by adding HEPES buffer to the 293T/NIH3T3 cell

growth medium at a final concentration of 10 m*M*. Once collected, vector supernatants should be kept on ice at all times and, if they are not being used immediately, stored in aliquots at −80°C. Avoid repeated freezing and thawing. The peak of vector particle production by transient transfection is on days 2 and 3 post-transfection. Therefore, the optimal time to collect the supernatant is 48 h after addition of fresh medium to the transfected cells.

6. Small (~2 to 5-mm-diameter) pellets should be visible after concentration by centrifugation. Pellets may not resuspend completely. Vortex overnight at low speed and 4°C to facilitate resuspension. Expect a 50−75% recovery following vector concentration.
7. When GFP is used as the reporter gene (87), it is useful to wait ~5 days before analyzing the transduced cells by flow cytometry. This will minimize the contribution of false positive signals due to pseudotransduction, which is the direct transfer of reporter protein present in the vector supernatants or incorporated into the vector particles to the target cells; this is particularly problematic for VSV-G-pseudotyped vectors (82,83). Note that it has also been shown that transgenes can be efficiently transiently expressed from unintegrated lentiviral vectors during this timeframe (100).
8. It should be possible to obtain $3-4 \times 10^6$ bone marrow cells from each 5-FU-treated mouse. The bone marrow cells can be used directly or further enriched for HSC/progenitors using various magnetic- or fluorescence-activated cell sorting procedures (91,92). When culturing bone marrow cells, keep the cell density at 0.5×10^6 cells/ml.
9. Detailed protocols for staining of murine hematopoietic cells and detection of cell surface antigens by immunofluorescence flow cytometric analysis may be found on the websites of the monoclonal antibody manufacturers.

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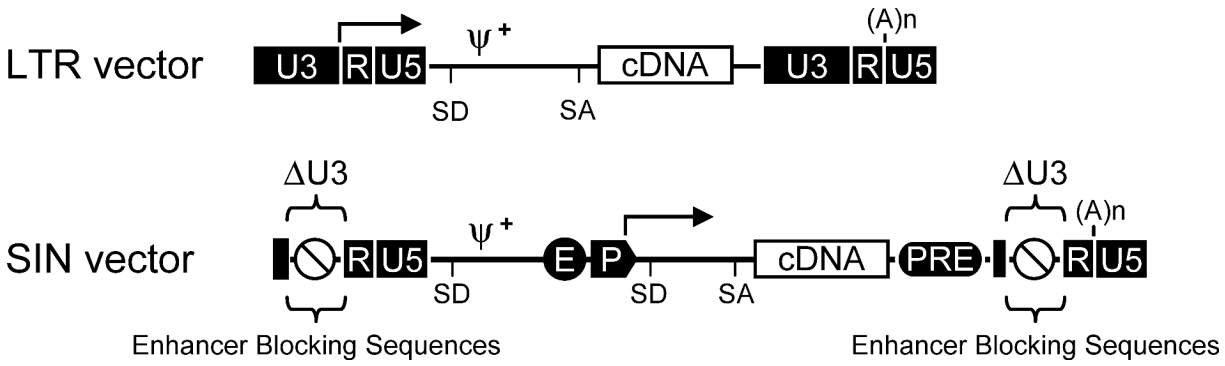

Figure 1.

Schematic representation of integrated forms of prototypical LTR and SIN retroviral vectors. For simplicity, gammaretroviral vectors are illustrated. Comparable lentiviral vectors contain additional components such as a central polypurine tract and the Rev response element involved in import of the preintegration complex into the nucleus and export of vector RNA into the cytoplasm, respectively (72,73). **(Top)** *LTR vector* The flanking intact LTRs comprise: U3, sequence unique to the 3' vector RNA and repeated in the integrated vector DNA; R, short sequence repeated at both termini of the vector RNA; and U5, sequence unique to the 5' vector RNA and repeated in the integrated vector DNA. Vector RNA transcripts initiate at the 5' boundary of the R region in the 5' LTR (arrow) and are polyadenylated ((A)n) at the 3' boundary of the R region in the 3' LTR. The packaging signal (ψ^+) allows the full-length vector RNA to be efficiently encapsidated into budding vector particles. The protein coding region (cDNA) of the gene of interest is expressed as a spliced transcript (SD, splice donor; SA, splice acceptor). **(Bottom)** *SIN vector* The enhancer-promoter elements are deleted from the U3 regions of the LTRs (ΔU3) and an internal enhancer (E)-promoter (P) is used to drive transgene expression. The transgene pre-mRNA has been optimized for improved splicing and 3' end formation by inclusion of an intron and a posttranscriptional regulatory element (PRE). Enhancer-mediated interactions with cellular promoters are modulated by inclusion of enhancer blocking sequences (⊘) in the deleted U3 regions of the LTRs.