HUMAN GENE THERAPY METHODS 24:228–240 (August 2013)
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DOI: 10.1089/hgtb.2012.190

RD2-MolPack-*Chim3,* a Packaging Cell Line for Stable Production of Lentiviral Vectors for Anti-HIV Gene Therapy

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Abstract

Over the last two decades, several attempts to generate packaging cells for lentiviral vectors (LV) have been made. Despite different technologies, no packaging clone is currently employed in clinical trials. We developed a new strategy for LV stable production based on the HEK-293T progenitor cells; the sequential insertion of the viral genes by integrating vectors; the constitutive expression of the viral components; and the RD114-TR envelope pseudotyping. We generated the intermediate clone PK-7 expressing constitutively *gag/pol* and *rev* genes and, by adding *tat* and *rd114-tr* genes, the stable packaging cell line RD2-MolPack, which can produce LV carrying any transfer vector (TV). Finally, we obtained the RD2-MolPack-*Chim3* producer clone by transducing RD2-MolPack cells with the TV expressing the anti-HIV transgene *Chim3*. Remarkably, RD114-TR pseudovirions have much higher potency when produced by stable compared with transient technology. Most importantly, comparable transduction efficiency in hematopoietic stem cells (HSC) is obtained with 2-logs less physical particles respect to VSV-G pseudovirions produced by transient transfection. Altogether, RD2-MolPack technology should be considered a valid option for large-scale production of LV to be used in gene therapy protocols employing HSC, resulting in the possibility of downsizing the manufacturing scale by about 10-fold in respect to transient technology.

Introduction

Since the first-ever lentiviral vector (LV) phase I clinical trial against AIDS in 2001, 53 phase I/II and two phase II/III trials using HIV-based LV for gene transfer protocols have undergone authorities' scrutiny. So far, LV have been used to genetically modify, mostly, hematopoietic stem cells (HSC) and lymphocytes to correct inherited and acquired disorders. Furthermore, significant progress has been recently made using stem-cell-based gene and cell therapy approaches to treat HIV-1 infection (Kitchen *et al.,* 2011). In this context, we have previously demonstrated that the therapeutic gene *Chim3*, an HIV-1 Vif-dominant negative factor, when delivered to target cells by means of Tat-conditional LV, inhibits HIV-1 replication and confers selective growth advantage to transduced cells (Porcellini *et al.,* 2009, 2010). *Chim3*-based anti-HIV gene therapy is therefore a promising therapeutic intervention, which requires an adequate clinical-grade manufacturing to be further tested in preclinical animal models or in patients. Unlike gamma retroviral vector (γRV), a limitation in LV field is the lack of clinical-grade stable packaging clones. Transient transfection is instead the current technology for pilot production of LV, which is impractical for very large-scale applications under a safety, cost, and reproducibility standpoint. In fact, this technology is expensive, is difficult to standardize and scale-up, and suffers from batch-to-batch variability and low reverse transcriptase fidelity (Laakso and Sutton, 2006).

Notably, the establishment of a γRV-equivalent packaging cell line for LV is more difficult than expected because of the expression of some viral proteins, which are toxic for producer cells, such as the HIV protease (Kaplan and Swanstrom, 1991; Krausslich, 1992; Krausslich *et al.,* 1993; Snasel *et al.,* 2000) and the VSV-G attachment protein (Burns *et al.,* 1993). To overcome this problem, in previous approaches, toxic genes were transcriptionally regulated by different systems, such as Tet (Kafri *et al.,* 1999; Klages *et al.,* 2000; Farson *et al.,* 2001; Xu *et al.,* 2001; Throm *et al.,* 2009), ecdysone (Pacchia *et al.,* 2001; Sparacio *et al.,* 2001), Rev (Klages *et al.,* 2000; Ni *et al.,* 2005), and the Tet–cumate combination (Broussau *et al.,* 2008), the removal of which has to be considered during downstream processing. Another important

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issue is the choice of the delivery vehicles used to integrate the genes encoding the vector components; most commonly, *gag-pol*, *rev*, and *env* genes are integrated by transient transfection of plasmid DNA (Carroll *et al.,* 1994; Corbeau *et al.,* 1996; Poeschla *et al.,* 1996; Yu *et al.,* 1996; Srinivasakumar *et al.,* 1997; Kaul *et al.,* 1998; Kafri *et al.,* 1999; Klages *et al.,* 2000; Farson *et al.,* 2001; Pacchia *et al.,* 2001; Sparacio *et al.,* 2001; Xu *et al.,* 2001; Ni *et al.,* 2005; Cockrell *et al.,* 2006; Broussau *et al.,* 2008). This method is known to suffer eventually from gene silencing and gene loss (Bestor, 2000), which can both jeopardize the long-term stability of the producer clone. Alternative methods have been applied to STAR and GPRG-TL-20 packaging cells, in which the *gag-pol* and *rev* genes were transduced by LTR-γRV and SIN-γRV, respectively (Ikeda *et al.,* 2003; Throm *et al.,* 2009), with the exception, in STAR cells, of the *env* gene introduced by plasmid transfection (Ikeda *et al.,* 2003).

On this premise, we conceived a new strategy to obtain RD2-MolPack-*Chim3,* a packaging clone for the stable production of LV carrying the *Chim3* transfer vector (TV). Notably, an equal number of concentrated RD114-TR pseudovirions transduces a higher proportion of progenitor $CD34^+$ stem cells compared with VSV-G pseudovirions.

Materials and Methods

Plasmids

Wild-type HIV-1 *gag*, *pol*, and *rev* genes were derived from the pCG719-pKL*gagpol* (hereafter named CMV-GPR) and pCG720-pK*rev* (CMV-*Rev*) plasmids, respectively (Cell Genesys, Inc.) (Fig. 1a, schemes 1 and 2). The viral genes were inserted into the Gateway pENTR4 shuttle vector (Invitrogen, Co.) in two distinct expression cassettes tail-to-tail oriented. The first cassette expresses the *gag* and *pol* genes, whereas the second one the *rev* and the selection marker hygromycin resistance (*hygro*) gene, which was cloned downstream an internal ribosome entry site (IRES) to allow bi-cistronic translation. The two expression units of the packaging construct were introduced into the *Xba*I site of the recombinant pSUB201 plasmid carrying an infectious adeno-associated viral genome (Samulski *et al.,* 1987). The resulting 5′ITR-CMV-*GagPol*-polyA-polyA-*hygro*-IRES-Rev-CMV-ITR3′ fragment was excised from the pSUB201 plasmid and inserted into the Gateway pENTR4 shuttle vector. The final recombinant hybrid baculo-AAV packaging vector (Baculo-AAV-GPR) (Fig. 1a, scheme 11) was obtained by the bacteriophage lambda site-specific recombination system between the pENTR4 shuttle entry vector, containing the two cassettes, and the BaculoDirect Linear DNA (BaculoDirect Baculovirus Expression Systems; Invitrogen, Co.). The polyhedrin gene of the baculo DNA was thereby replaced with the GPR double cassette during homologous recombination.

The pABCMV-*Rep78* plasmid (CMV-AAV-*Rep78*) was obtained by cloning the AAV-*Rep78* ORF under the CMV IE promoter of the pABS.43 vector (Recchia *et al.,* 2004) (Fig. 1a, scheme 3). The pMD.G plasmid (CMV-*VSV-G*) encodes the vesicular stomatitis envelope glycoprotein (VSV-G) (Fig. 1a, scheme 4). The pCCLsin.PPT.hPGK.*eGFP*.WPRE.Amp (SIN-*eGFP*) TV and the second-generation packaging pCMV-ΔR8.74 (CMV-GPRT) construct encoding the HIV *gag, pol, rev,* and *tat* genes were kindly provided by L. Naldini (Tiget; HSR) (Fig. 1a, schemes 5 and 6). The second-generation PΔN-*Chim3* TV was previously described (Porcellini *et al.,* 2009, 2010) (Fig. 1a, scheme 7). The PΔN-*eGFP* was obtained by substituting the *Chim3* ORF with the *eGFP* transgene. The SIN-*RD114-TR*-IN-RRE vector (Fig. 1a, scheme 8) was constructed by using the *RD114-TR* ORF excised from the pCMV-*RD114-TR* plasmid (CMV-*RD114-TR*) (Sandrin *et al.,* 2002) (Fig. 1a, scheme 9). The CMV-*RD114*-TR-IN-RRE cassette of the SIN-*RD114-TR*-IN-RRE vector contains the rabbit *β-globin* intron derived from the CMV-*RD114-TR* vector in which the 230 bp RRE element, polymerase chain reaction (PCR) amplified as described in the Analytical PCR analysis section, was integrated into the *Sca*I site of the *β-globin* intron. This cassette was cloned into the *Mlu*I site of the SIN-poly*Mlu*I vector, a modified version of the SIN-*eGFP* vector in which the hPGK-*eGFP* cassette was removed and substituted with the *Eco*RV-*Mlu*I-*Sma*I-*Mlu*I-*Not*I-*Sac*I-*Bgl*II-*Bam*HI-*Sal*I poly-linker. The SIN-*Tat* vector was constructed by inserting the *tat* gene, derived from the CMV-GPRT plasmid, into the pIRESpuro3 (Clontech Laboratories, Inc.), and then by cloning the bi-cistronic CMV-*Tat*-IRES-puro cassette into the *Mlu*I site of the SIN-poly*Mlu*I vector (Fig. 1a, scheme 10).

Cells

Spodoptera frugiperda (Sf9) insect cells (Invitrogen, Co.) were grown in TC-100 medium (Invitrogen, Co.) supplemented with 10% fetal calf serum (FCS; EuroClone Ltd.) and a combination of penicillin–streptomycin and glutamine (PSG) at 27°C in a non-CO_2 incubator. Human embryo kidney 293T (HEK-293T) cells and its derivatives were propagated in Dulbecco's modified Eagle's medium supplemented with 10% FCS and PSG. SupT1 and CEM A3.01 T cells were grown in RPMI 1640 supplemented with 10% FCS and PSG. Human $CD34^+$ HSC and neonatal leukocytes were purified from UCB centrifugation on a Ficoll-Hypaque gradient (Lymphoprep; Nycomed Pharma AS) after approval of the local institutional review board and written informed consent. $CD34^+$ HSC were isolated from the UCB mononucleated cells by positive selection using CD34 MicroBeads Kit and MiniMACS Separator Columns (Miltenyi Biotec). $CD34^+$ cells purity was >92%, as established by FACS analysis (FACSCalibur BD Bioscience) using the anti-CD34-PE antibody (BD Pharmingen) and the FlowJo software (Tree Star, Inc.). $CD34^+$ cells and neonatal leukocytes were prestimulated for 48 hr in a cytokine cocktail and maintained in the same medium during transduction and cultivation as previously described (Porcellini *et al.,* 2009, 2010).

Baculovirus production and baculo-GPR infection of HEK-293T cells

Baculovirus carrying the recombinant hybrid Baculo-AAV-GPR DNA genome (Fig. 1a, scheme 11) was produced following the BaculoDirect method using the Gateway-adapted Baculovirus DNA system (Invitrogen, Co.). After three cycles (p3) of recombinant baculovirus amplification in Sf9 cells, we checked the titer and the potential recombination events of the hybrid baculo-AAV DNA by plaque assay and Southern blot of viral genomic DNA (gDNA), respectively. The titer corresponded to 6×10^{10} pfu/ml, and a single sharp band was revealed by Southern blot, demonstrating no recombination

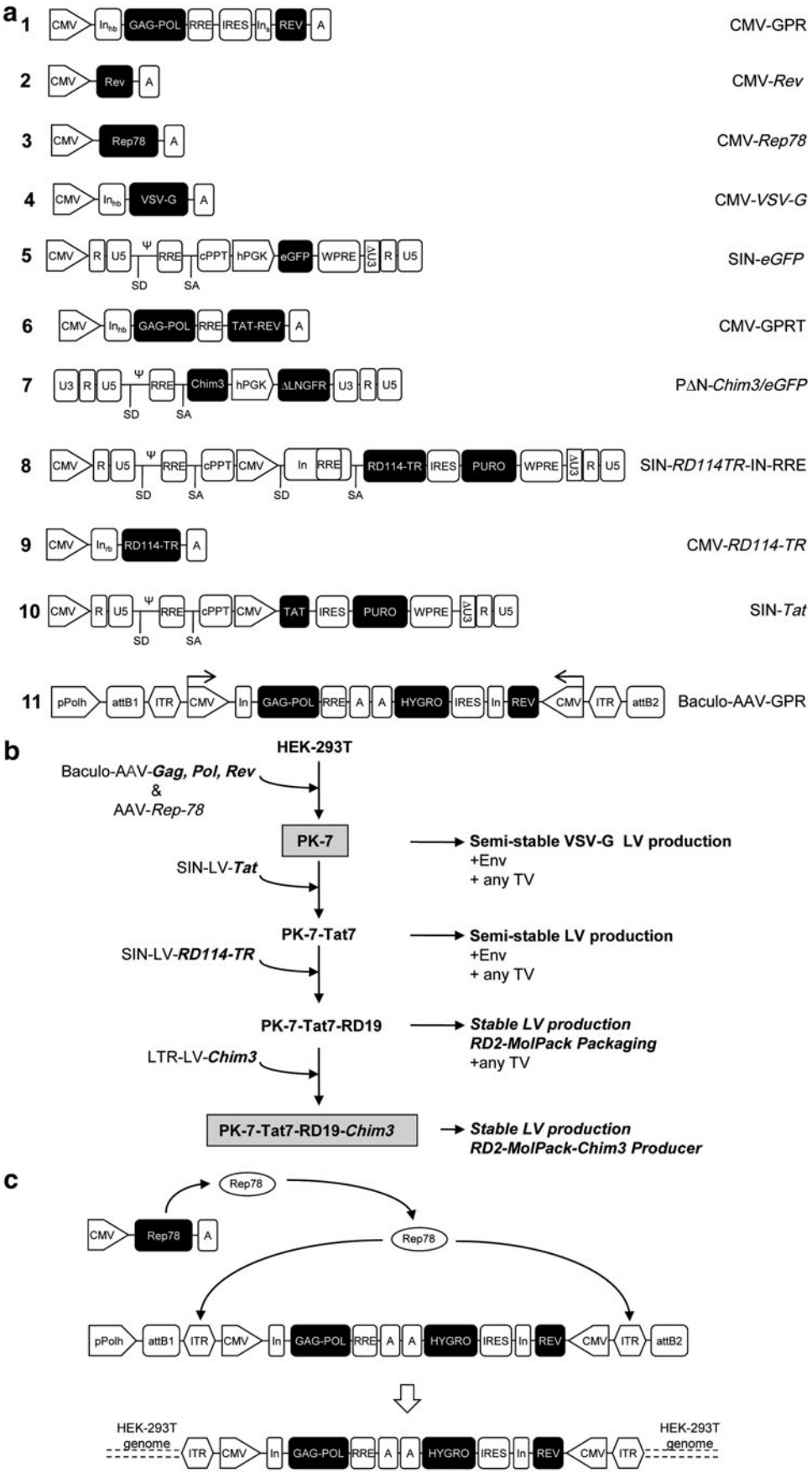

FIG. 1. Strategy of RD-MolPack development. **(a)** Schemes of plasmids and vectors used in this study. pPolh, polyhedrin promoter; attB1, attachment B1; ITR, inverted terminal repeat; CMV, cytomegalovirus promoter; In, intron; RRE, *rev* responsive element; A, polyA sequence; IRES, internal ribosome entry site; SD, splice donor; SA, splice acceptor; Ψ, packaging signal; WPRE, woodchuck hepatitis post-transcriptional regulatory element; cPPT, central polypurine tract; hPGK, human phosphoglycerate kinase promoter. **(b)** Flow chart of the development strategy of PK-7 and RD-MolPack-*Chim3* packaging cells. **(c)** Cartoon of the Rep78-mediated integration of the AAV-GPR vector into HEK-293T host genome. AAV Rep78 promotes the excision of the ITR-flanked AAV-GPR cassette and facilitates the integration into human chromosomes in either single or concatameric configuration.

events during virus amplification (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/hgtb). To obtain the PK-7 clone, the following parameters were individually optimized: (1) dose and time of AAV-*Rep78* plasmid DNA transfection; (2) multiplicity of infection (MOI) of rh-baculo-AAV; (3) cloning strategy for infected HEK-293T cells. The best overall output was obtained by transfecting 1.5×10^6 cells with 4 μg of AAV-*Rep78* expression plasmid and 24 hr afterward infecting the cells with the recombinant Baculo-AAV-GPR (MOI = 1,000). After 4 days, a total of 5×10^5 cells were seeded in twenty-two 10 cm dishes in the presence of hygromycin (100 μg/ml) at serially diluted concentrations. The 22 dishes were screened by p24Gag enzyme-linked immunosorbent assay (ELISA). Only the dishes seeded with 3.7×10^4 cells/dish contained 40 colonies and released detectable p24Gag. Three of the 40 colonies were further characterized.

LV production, concentration, and titration

LV were obtained by transient co-transfection of HEK-293T cells with the following plasmids: the packaging constructs CMV-GPR (third-generation) [or CMV-GPRT (second-generation)], the VSV-G or RD114-TR envelope constructs, and the third-generation SIN-*eGFP* or the second-generation PΔN-*Chim3* or PΔN-*eGFP* TV. LV produced from PK-7 clone were generated by co-transfecting the Env plasmid and the TV, whereas LV produced from the PK-7-Tat7-RD clones, only the TV. Supernatants were harvested 24 hr after transfection and filtered through 0.45 μm filters, and, when indicated, concentrated 100-fold by low-speed centrifugation (4,000 rpm at 4°C for 16 hr in a Multifuge 32-R). The viral pellets were resuspended in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum and frozen at −70°C (Strang *et al.,* 2004). Titer was calculated on different cell lines by one or two cycles of spinoculation at 1,240 *g* for 45 min in the presence of polybrene 8 μg/ml (Sigma-Aldrich); $CD34^+$ HSCs were transduced for 24 hr on retronectin-coated plates (Takara Bio) as previously described (Porcellini *et al.,* 2009, 2010). Transduction efficiency was monitored by flow cytometry analysis (FACS Calibur BD Bioscience) of eGFP (SIN-*eGFP*) or ΔLNFGR expression (PΔN-*Chim3*), as previously described (Porcellini *et al.,* 2009, 2010).

Titer-based protocol for screening of LV producer clones

All PK-7 derivative subclones were screened by calculating the LV titer of their supernatants on SupT1 cells by a small-scale transduction protocol. Briefly, 6×10^4 cells/well were seeded in 48-well plate and 24 hr later co-transfected with the plasmids needed to obtain LV. Forty-eight hours after transfection, 200 μl of culture supernatants was used to transduce 3×10^4 SupT1 cells/well seeded at 7.5×10^4 cells/ml. The titer threshold score was imposed $\geq1\times10^2$ TU/ml.

RCL test

RCL test was carried out exactly as described by Cornetta *et al.* (2011). Briefly, RD2-MolPack-*Chim3* supernatant (1.75 liters) was obtained by seven independent harvests from 10 T162 flasks/harvest (total 70 T162 flasks). The supernatants of each harvest were concentrated, and 1 μg p24Gag LV/harvest was used as test article in the amplification phase. The supernatants of the seven independent amplification phases were then used in seven independent indicator phases. The endpoints of the test were evaluated by the immunological assay p24Gag ELISA and the molecular psi-gag recombination PCR assay as described in the specific sections.

Analytical PCR analysis

The 868 bp AAV-*Rep78* amplicon was obtained by using 1 pg of control plasmid CMV-*Rep78* DNA and 300 ng of gDNA of PK-7 cells; the following set of primers: AAV-*Rep78* forward, 5′-CGG GCT GCT GGC CCA CCA GG-3′, and AAV-*Rep78* reverse, 5′-ATG CCG GGG TTT TAC GAG ATT GTG-3′; and the following PCR conditions: 98°C for 7 min, 30 cycles of 94°C for 30 sec, 66°C for 30 sec, and 72°C for 1.5 min.

The 230 bp RRE amplicon was obtained by using 1 ng of SIN-*eGFP* vector as DNA template; the following set of primers: RRE-forward, 5′-AGT ACT GGA GCT TTG TTC CTT GGG-3′, and RRE-reverse, 5′-AGT ACT AAA TCC CCA GGA GCT G-3′; and the following PCR conditions: 98°C for 7 min, 30 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec.

To establish orientation of the two integrated GPR cassettes, PCR amplification was performed on 300 ng of gDNA of PK-7 cells with the set of primers: *Rev* forward, 5′- CTT GAG GAG GTC TTC GTC GC-3′; *β-glob.* reverse, 5′-CCC TGT TAC TTC TCC CCT TCC-3′; *Rev* forward nested, 5′-TGT CTC CGC TTC TTC CTG CC-3′; and *β-glob.* reverse nested, 5′-TTA ACC ATA GAA AAG AAG GGG-3′; and the following PCR conditions: 94°C for 2 min, 35 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1.5 min.

PCR conditions for the detection of putative psi-gag recombination events in the RCL test were carried out exactly as described in Cornetta *et al.* (2011).

Vector copy number quantification by quantitative PCR

The vector copy number (VCN) of the integrated vector was established by Q-TaqMan PCR using an ABI Prism 7900 FAST instrument (Applied Biosystems) and analyzed by SDS 2.3 software (Applied Biosystems). PCR conditions were the following: 2 min at 50°C and 15 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. gDNA was amplified by using the primers and probes of Supplementary Table S1.

HIV p24Gag ELISA

Physical LV production estimate was obtained by measuring p24Gag with the Alliance HIV-1 p24 Antigen ELISA kit (Perkin Elmer Life and Analytical Sciences, Inc.) following manufacturer's instructions in culture supernatants, with the assumption that 1 ng p24Gag corresponds to 1×10^7 physical particles (pp).

Ligation-mediated PCR

gDNA was extracted from PK-7 cells by QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions and digested with *Bgl*II and *Bam*HI at 37°C overnight. Ligation of an adaptor 76-bp oligonucleotide linker compatible with the 5′-GATC-3′ ends was performed under standard conditions.

Ligation-mediated (LM)-PCR was carried out using the nested primers of Supplementary Table S2. Two rounds of LM-PCR were carried out using AmpliTaq Gold DNA Polymerase (Applied Biosystems), each comprising 30 cycles (95°C for 30 sec, 52°C for 30 sec, 72°C for 2 min). PCR amplicons were cloned using the TOPO cloning kit (Invitrogen, Co.) and plasmid colonies carrying inserts of approximately 100–200 bp were selected for sequencing. Sequence homologies were identified by BLAST search, NCBI, as previously described (Maruggi *et al.*, 2009).

Southern and Northern blot assays

gDNA was isolated by the QIAamp Mini kit (Qiagen GmbH) according to manufacturer's instructions. Baculovirus DNA was extracted from viral particles by the QIAamp DNA micro kit (Qiagen). After overnight digestion with the indicated restriction enzymes, 10 μg/sample was run on 0.8% agarose gel, blotted by Southern capillary transfer onto nylon membranes (Duralon), and then hybridized to 1×10^6 dpm/ml of ^{32}P-random primed labeled of either 600 bp CMV, 11 kb GPR, 250 bp *Tat*, 600 bp *Chim3*, or 500 bp *RD114-TR*-specific probe, in PerfectHyb PLUS hybridization buffer.

Total RNA was isolated by the TRIzol reagent (Ambion; Life Technologies Ltd.) according to manufacturer's instructions. About 5 μg/sample was run on denaturing formaldehyde/MOPS 1.0% agarose gel, blotted by capillary transfer onto nylon membranes (Duralon), and then hybridized to 2×10^6 dpm/ml of ^{32}P-random primed labeled of either 250 bp *Tat*, 500 bp *RD114-TR*, or 270 bp ψ-specific probe, in PerfectHyb PLUS hybridization buffer.

After extensive washes, the membranes were exposed to X-ray films at −80°C or to PhosphorImager (Molecular Dynamics).

Western blot analysis

Cellular and viral proteins, the latter derived from isolated cell-free viral-like particles (VLPs) or LV, were prepared and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis as previously described (Porcellini *et al.*, 2009, 2010). The anti-HIV serum, obtained from an AIDS patient, was used at 1:1,000 dilution; the anti-transmembrane (TM) RD114-TR rabbit serum, kindly provided by F.L. Cosset (Inserm) (Sandrin *et al.*, 2004) was used at 1:1,000 dilution; the anti-surface RD114-TR MoAb, generated by Areta International, was used at 1:50 dilution; the HIV-1 Rev MoAb (Rev-6, sc-69730), the affinity-purified rabbit polyclonal anti-YY1 antibody (C-20, sc-281) (Santa Cruz Biotechnology, Inc.), and the mouse anti-p24Gag (Acris Antibodies) were used at 1:200, 1:1,000, and 1:500 dilution, respectively. HIV-1 Tat MoAb (1D9) was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD) from Dr. Dag E. Helland and used at 1:1,000 dilution. The antihuman and antimouse secondary antibodies were diluted 1:10,000, whereas the antirabbit 1:20,000.

Fluorescence in situ *hybridization*

Metaphase chromosomes were obtained by treating PK-7 cells with 10 μg/ml colchicine (Sigma-Aldrich) for 2 hr at 37°C. After standard phosphate buffer saline (PBS) washing, cells were kept in 75 m*M* KCl hypotonic solution for 6 min at room temperature, fixed with four washes of methanol/acetic acid (3:1), and then spread on a glass slide. Samples were denatured in 70% formamide for 2 min at 72°C, dehydrated by cold 70%, 85%, and 95% ethanol washes, and then air-dried. The 13 kb plasmid DNA containing the GPR-cassette-specific probe was labeled using the Random Primed DNA Labelling Kit (Roche Applied Science) with SpectrumOrange-dUTP (Vysis, Inc.), whereas the control 30 kb cosmid DNA containing the human *hox4* gene was labeled using the fluorescence *in situ* hybridization (FISH) *Bright* Nucleic Acid Labelling kit (Kreatech Biotechnology). Hybridization was performed by incubating 1.25 μg of each probe in 250 μl of 50% formamide, 2×SSC, 10% dextran sulfate, and 12.5 μg of human C_0T-1 DNA hybridization buffer (Invitrogen, Co.). Samples were coated with denatured probes for 10 min at 75°C, covered with Parafilm M, and incubated overnight at 37°C in a moist chamber. Samples were washed once in 0.4×SSC, pH 7, at 72°C for 2 min; once in 4×SSC, pH 7, containing 0.0025% Tween-20 for 30 sec; and twice in PBS 1× for 1 min at room temperature. Slides were counterstained with 0.02 μg/μl of 49,6-diamidino-2-phenylindole (DAPI; Sigma). Visualization and photographic images were taken with a Nikon 80i upright microscope (Nikon Instruments S.p.A.) using the FITC (green) and spectrum orange (orange) filter illumination. Images were processed with Genikon software (Nikon).

Results

Development of the intermediate PK-7 clone

To obtain RD2-MolPack packaging cells, we developed several HEK-293T-derived intermediate clones (Fig. 1b). The first one, PK-7, was created by stable integration of HIV-1 *gag-pol* and *rev* genes by means of the recombinant hybrid baculo-AAV vector (rhBaculo-AAV-GPR) (Fig. 1a, scheme 11) in the transient presence of AAV-Rep78 protein (Fig. 1c). Only three of the 360 grown-up, PK-7, −17, and −18 clones, expressed p24Gag above the 0.1 ng/ml arbitrary threshold, indicating a low level of tolerance for the constitutive expression of Gag, Pol, and Rev proteins. Southern blot and quantitative PCR (Q-PCR) analyses revealed that each clone contains two copies of the correct-in-size vector (Fig. 2a), which produces HIV-1 viral proteins rightly processed and in the right relative proportion in both cell and VLP extracts as visualized by Western blot analysis (Fig. 2b). For further genetic manipulation, we selected the PK-7 clone based on cell morphology, growth capabilities, viability, p24Gag production, and titer of the LV produced after transfecting the VSV-G plasmid and the SIN-*eGFP* TV, which correspondes at the best value to 1.1×10^7 TU/ml (mean $3.6\times10^6\pm0.6$ SEM TU/ml, $n=12$) (Supplementary Table S3) when calculated on SupT1 cells. Next, we spotlighted the break point of the ITR-GPR cassette integration on the chromosome 2q32.1 locus by LM-PCR technology (Fig. 2c), confirming this result by FISH using the specific GPR probe and, as internal control, the human *HOX4* gene probe, which is known to map on the chromosome 2q31.2 locus (Fig. 2d). We demonstrated, by using an appropriate primer set design in nested PCR, that the two copies of packaging construct are integrated in tail-to-head orientation (Fig. 2e). Sequence analysis of the amplicon encompassing the tail-to-head junction showed that a 910 bp fragment got lost likely during integration (Fig. 2e). To exclude integration of residual AAV-*Rep78* plasmid

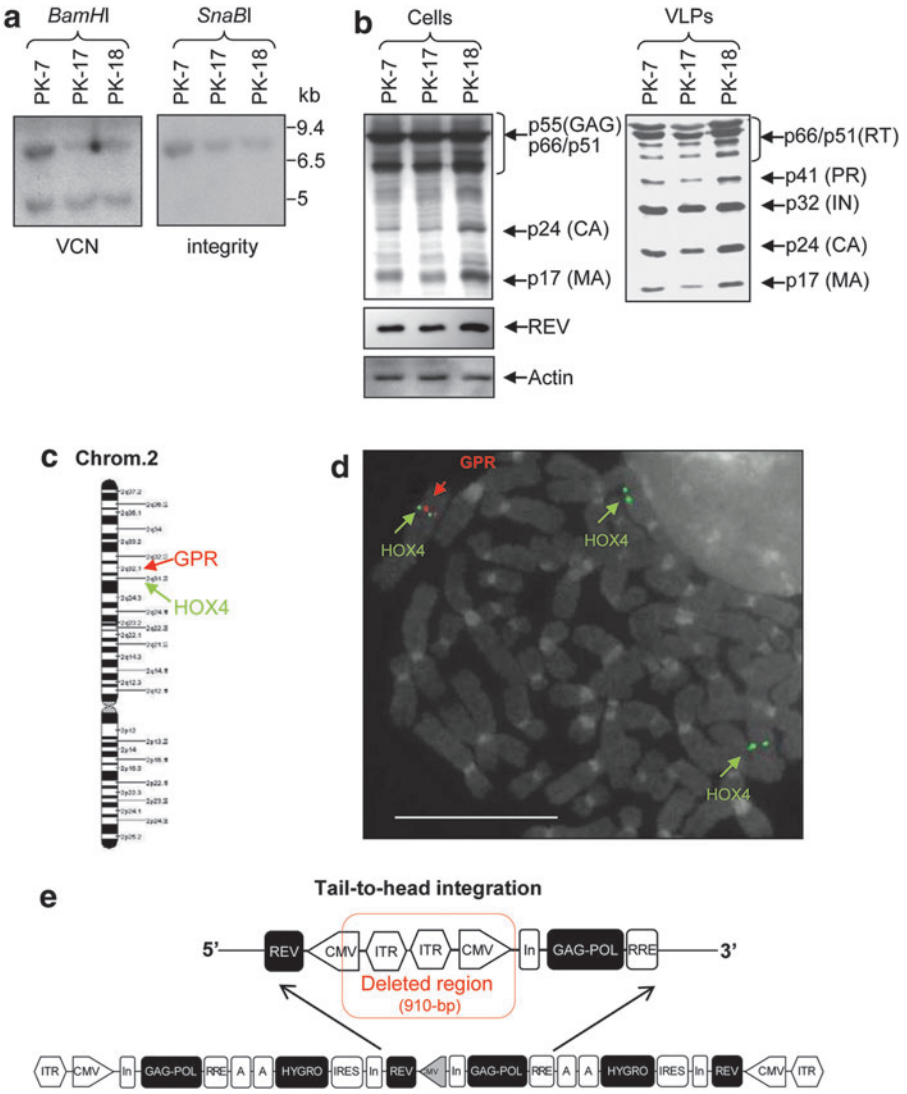

FIG. 2. Characterization of PK-7 clone. **(a)** Southern blot analysis of the AAV-GPR vector integration. About 10 μg/sample of gDNA derived from PK clones was digested with *Bam*HI and *Sna*BI restriction enzymes and hybridized with the specific probe to establish the VCN and the integrity of the cassette (integrity). **(b)** Western blot analysis of the viral proteins. Left panel (cells): 50 μg/sample of cell extract obtained from the PK clones was hybridized to an anti-HIV human serum-recognizing HIV-1 proteins. The membrane was sequentially hybridized with anti-Rev and anti-actin antibodies. Right panel (VLPs): 30 ng p24Gag-equivalent/sample of VLP produced by PK clones was processed similarly to the cellular extracts. **(c)** Schematic mapping of the GPR cassette integration by LM-PCR technique indicating the break point in the long arm of chromosome 2 at the 2q32.1 location and the known location of the *HOX4* gene. **(d)** Co-localization of the AAV-GPR cassette and the *HOX4* gene into chromosome 2. FISH analysis of PK-7 metaphase chromosomes was carried out by using *Gag*-specific (red) and *HOX4*-specific (green) probes. As HEK-293T cells are triploid, *HOX4* signal is detected on the three chromosomes 2. **(e)** Schematic representation of the rearrangement of the two GPR-integrated cassettes in the PK-7 clone and their tail-to-head orientation. FISH, fluorescent in situ hybridization; LM-PCR, ligation-mediated polymerase chain reaction; VCN, vector copy number; VLP, viral-like particles. gDNA, genomic DNA.

DNA, we carried out AAV-*Rep78*-specific PCR on PK-7 gDNA detecting no amplification (Supplementary Fig. S1b). To demonstrate genetic and functional stability of PK-7 clone over time, we cultivated the cells in the presence or absence of hygromycin for 350 days and measured p24Gag production on a per cell basis (Table 1). Next, we established that PK-7 cells maintain long-term functionality by cotransfecting passage 60 (p60) and p102 cells with the *VSV-G* plasmid and SIN-*eGFP* TV either in the presence or in the absence of antibiotic (Table 1). As in both passages and conditions titer values were similar (1.3–3.2$\times 10^6$ TU/ml), we avoided the use of hygromycin in further experiments. Furthermore, to determine whether PK-7 clone is suitable for semi-stable LV production, we transfected in parallel PK-7 and HEK-293T cells with the plasmids necessary, in each cell type, for LV production. We measured on SupT1 and CD34$^+$ cells the titer of second-generation LV (PΔN-*Chim3*) and third-generation LV (SIN-*eGFP*) produced from the two cell types. The titer of PK-7-derived PΔN-*Chim3* LV is comparable to the HEK-293T-derived LV, whereas the titer of PK-7-derived

Table 1. Stability of PK-7 Clone

	p24Gag ng/10^6 cells/day	
Passage	*Hygro*	*No Hygro*
p2	10.0	8.10
p6	7.00	4.80
p10	11.4	4.00
p16	5.00	4.80
p20	9.30	5.20
p24	7.20	6.50
p28	9.20	4.40
p32	6.20	8.80
p36	11.0	9.60
p40	18.0	10.0
p44	4.30	8.40
p48	37.5	3.80
p52	7.00	3.20
p56	11.0	6.70
p60	*19.0*	*4.40*
p64	*22.0*	*18.7*
p68	15.2	8.00
p72	16.5	4.90
p76	17.8	7.60
p80	30.0	11.0
p84	27.0	8.40
p88	23.2	3.50
p92	23.0	7.50
p98	16.7	1.30
p102	19.0	3.80
Mean ± SD	15.3 ± 8.4	6.70 ± 3.5
	Titer (TU/ml)[a]	
p60	3.2×10^6	2.0×10^6
p102	2.7×10^6	1.3×10^6
	p24Gag (ng/ml)[a]	
p60	86	38
p102	80	13
	Infectivity (TU/ng p24Gag)[a]	
p60	4.2×10^4	5.2×10^4
p102	3.3×10^4	1.0×10^5

[a]Potency values of VSV-G-pseudotyped lentiviral vectors (LV) produced after transfection of PK-7 cells with SIN-*eGFP* and *VSV-G* plasmids and tested on SupT1 cells 3 days after transduction.

Table 2. Potency of VSV-G-Pseudotyped Second- and Third-Generation Lentiviral Vectors Produced from PK-7 Clone

		SupT1	*$CD34^+$*
Vector	*Producer*	*Titer (TU/ml)*	
PΔN-Chim3	PK-7[a]	5.8×10^6	3.5×10^4
	HEK-293T[b]	1.0×10^7	3.9×10^4
		p24Gag (ng/ml)	
	PK-7	166	166
	HEK-293T	237	237
		Infectivity (TU/ng p24Gag)	
	PK-7	3.5×10^4	2.0×10^2
	HEK-293T	4.2×10^4	1.6×10^2
SIN-eGFP		*Titer (TU/ml)*	
	PK-7[a]	5.7×10^6	2.4×10^4
	HEK-293T[b]	5.6×10^7	3.0×10^5
		p24Gag (ng/ml)	
	PK-7	45.0	45.0
	HEK-293T	350	350
		Infectivity (TU/ng p24Gag)	
	PK-7	1.2×10^5	5.3×10^2
	HEK-293T	1.6×10^5	8.6×10^2

[a]VSV-G-pseudotyped LV were produced after transfection of PK-7 cells with either the SIN-eGFP or the PΔN-Chim3 and VSV-G envelope plasmids.
[b]VSV-G-pseudotyped LV were produced after transfection of HEK-293T cells with CMV-GPR, SIN-eGFP (or PΔN-Chim3), and VSV-G envelope plasmids. LV were tested on target cells after 3–5 days from transduction.

SIN-*eGFP* LV is 1-log lower than the HEK-293T-derived LV (Table 2).

Development of RD2-MolPack stable packaging cells

To generate RD2-MolPack packaging clone (Fig. 1b), we carried out stable integration of the *tat* gene into PK-7 cells by means of SIN-*Tat* LV delivery (Fig. 1a, scheme 10). Cells were SIN-*Tat* transduced, puromycin selected, and then cloned by limiting dilution. We measured Tat expression by Western blot in 5 of the 11 screened clones producing ≥5 ng p24Gag/1×10^6 cells. Both PK-7-Tat5 and PK-7-Tat7 clones showed elevated expression of Tat, released 110 and 130 ng p24Gag/1×10^6 cells, and contained 12 and 6 copies of SIN-*Tat*, respectively (Supplementary Fig. S2). After small-scale transfection of the TV and *vsv-g* plasmids, we selected PK-7-Tat7 to integrate the RD114-TR envelope because, in view of a similar titer (Supplementary Table S4), the level of p24Gag of PK-7-Tat7 is lower and the number of copies of SIN-*Tat* lower than those of PK-7-Tat5. Next, we stably integrated the *rd114-tr* gene into PK-7-Tat7 cells by SIN-*RD114-TR*-IN-RRE LV transfer. The rationale of the complicated design of this vector is described elsewhere (Stornaiuolo *et al.*, in preparation). After SIN-*RD114-TR*-IN-RRE LV transduction, PK-7-Tat7 cells were cloned by limiting dilution, and three out of nine selected clones, PK-7-Tat7-RD3, -RD12, and -RD19, were screened as done for PK-7-Tat cells. We decided on PK-7-Tat7-RD19 clone because it produces the highest titer (3×10^5 TU/ml) (Supplementary Table S5) and the highest amount of RD114-TR transmembrane and surface subunits, and contains the highest copies of SIN-*RD114-TR* vector (Supplementary Fig. S2b). We named the PK-7-Tat7-RD19 clone, RD2-MolPack, which is a stable packaging cell line for the production of any LV.

Development of RD2-MolPack-Chim3 *producer clone*

To construct the RD2-MolPack-*Chim3* producer clone from the packaging cells, we stably integrated the PΔN-*Chim3* TV by

Table 3. Potency of Lentiviral Vectors Produced from RD2-MolPack-Chim3 Clones

Clones	SupT1	CD34+
	Titer (TU/ml)	
RD2-MolPack-Chim3.2	1.9×10^5	1.1×10^5
RD2-MolPack-Chim3.3	7.2×10^5	2.6×10^5
RD2-MolPack-Chim3.14	5.0×10^5	$\mathbf{5.1\times10^5}$
RD2-MolPack-Chim3.25	1.2×10^6	$\mathbf{1.0\times10^6}$
PK-7[a]	1.5×10^4	2.5×10^4
HEK-293T[b]	2.0×10^5	8.6×10^4
	p24Gag (ng/ml)	
RD2-MolPack-Chim3.2[a]	112	112
RD2-MolPack-Chim3.3	120	120
RD2-MolPack-Chim3.14	127	**127**
RD2-MolPack-Chim3.25	95.0	**95.0**
PK-7	128	128
HEK-293T	275	275
	Infectivity (TU/ng p24Gag)	
RD2-MolPack-Chim3.2	1.7×10^3	9.8×10^2
RD2-MolPack-Chim3.3	6.0×10^3	2.1×10^3
RD2-MolPack-Chim3.14	4.0×10^3	$\mathbf{4.0\times10^3}$
RD2-MolPack-Chim3.25	2.4×10^4	$\mathbf{1.0\times10^4}$
PK-7	1.2×10^2	1.9×10^2
HEK-293T	7.2×10^2	3.1×10^2

Bold indicates the selected clones.
[a]LV were produced after transfection of PK-7 cells with the PΔN-*Chim3* transfer vector and RD114-TR envelope plasmids.
[b]LV were produced after transfection of HEK-293Tcells with CMV-GPR, PΔN-*Chim3* transfer vector, and *RD114-TR* envelope plasmids. LV were tested on target cells 5 days after transduction.

transduction of the VSV-G pseudotyped Chim3 LV. After the standardized screening protocol, we analyzed the potency of LV produced from four clones, RD2-MolPack-*Chim3.2, -Chim3.3, -Chim3.14,* and *-Chim3.25,* on SupT1 and CD34+ HSC (Table 3). We selected RD2-MolPack-*Chim3.14* and *-Chim3.25* clones for further characterization because they provided the best titer on CD34+ cells (5.1×10^5 and 1×10^6 TU/ml, respectively) (Table 3). Remarkably, the potency of the vectors produced from both clones is higher than that of the corresponding RD114-TR pseudovirions produced by transient transfection (Table 3). We then checked the integrity of the exogenous genes into eight producer cells by Southern and Western blot analyses demonstrating that each gene maintains its integrity in all, but one (*Chim3* TV in the *-Chim3.10* clone), clones (Fig. 3a, right panel), and that the amount and size of the corresponding proteins are correctly detected in all clones (Fig. 3b). These findings indicate that the use of SIN-LVs to integrate the viral components in an LV packaging cell itself is feasible and safe when the integration of the viral genes occurs in a sequential order. To further address the safety of RD2-MolPack-*Chim3.25* cells, we carried out Northern blot analysis looking for potential mobilization of the *RD114-TR* and *Tat* expression cassettes, which could be generated by an host promoter reading through the 5′LTR of the vector and, so doing, incorporating the packaging signal. We did not detect aberrant transcripts using the psi probe, suggesting no mobilization going on (Supplementary Fig. S3). Additionally, we verified the possible mobilization of *RD114-TR, Tat, E1A,* and *SV40 LTA* in CD34+ transduced cells by Q-PCR for the corresponding potentially integrated DNA sequences, detecting no amplification (Supplementary Table S6). In particular, we carried out a deep analysis of the possible mobilization of the SIN-LV encoding the *Tat* and *RD114-TR* genes by Q-PCR. We first calculated the expected frequency of the two SIN-LV inside the *Chim3*-LV based on the number of integrated copies and the known mobilization frequency of SIN-LV (1:1,000–3,000) (Hanawa *et al.,* 2005) (Supplementary Table S7). We then calculated the true mobilization frequency into viral particles by measuring the percentage of the specific *Tat* and *RD114-TR* RNA respect to that of *Chim3* after *in vitro* retrotranscription (Supplementary Table S7). We measured a frequency of 0.47% for *RD114-TR* and 0.99% for *Tat* that takes into account the contribution of both the specific gRNA and mRNA encapsidated into the viral particles. Finally, we calculated the expected and the real number of mobilized copies present in CD34+ cells transduced >90%, detecting no signal for either SIN-LV, indicating that the amount of retrotranscribed RNA contained into the LV corresponds mostly to mRNA or small, nonfunctional gRNA sequences (Supplementary Table S7). We also verified the functional stability of RD2-MolPack-*Chim3.25* clone as previously done for PK-7 cells. The RD2-MolPack-*Chim3.25* clone persists in continuous culture with ≥80% viability for longer than 3 months, producing steady LV titer until p20 (87 days) that corresponds to slightly less than 3 months (Supplementary Table S8). Finally, we demonstrated the absence of RCL on a medium scale production lot equivalent to 7,146 ng p24Gag LV produced by RD2-MolPack-*Chim3.25* cells by two virus detection assays: the immunological p24Gag ELISA (Supplementary Table S9) and the molecular analytical PCR for possible psi-gag recombination (Supplementary Fig. S4).

*RD2-MolPack-*Chim3 *LV transduce more efficiently CD34+ cells than VSV-G pseudotyped* Chim3 *LV produced transiently*

To assess the transduction efficiency in CD34+ cells of RD2-MolPack-*Chim3* LV compared with VSV-G pseudotyped LV produced by standard transfection, we performed a comparative study using equal amount of pp of each type of LV, carrying the *Chim3* TV. LV were concentrated by low-speed centrifugation (Strang *et al.,* 2004) and then 73, 7.3, and 0.73 ng p24Gag of each LV were delivered to CD34+ cells and CEM A3.01, as control cells. After 5 and 14 days, transduction values of PΔN-*Chim3* LV were calculated (Table 4). Notably, we observed that 73 ng p24Gag of RD114-TR pseudovirions transduces 1.93-fold more CD34+ cells compared with the same amount of pp of VSV-G pseudovirions after 5 days from transduction. The difference increases with the decrement of the amount of pp used (3.2-fold more with 7.3 ng and 8.2-fold more with 0.73 ng p24Gag). In contrast, in CEM A3.01 cells the ratios of transduction efficiency of RD114-TR- versus VSV-G-pseudovirions are 0.94 (73 ng), 0.52 (7.3 ng), and 0.24 (0.73 ng). These data clearly establish the power of RD114-TR-pseudovirions in HSC, but not in CEM A3.01 cells. Again, in CD34+ cells, 2-log less RD114-TR LV (0.73 ng p24Gag) than VSV-G LV (73 ng p24Gag) pp is sufficient to obtain equivalent VCN (1.05 and 0.95, respectively) whereas, in CEM A3.01 cells, 1-log more RD114-TR

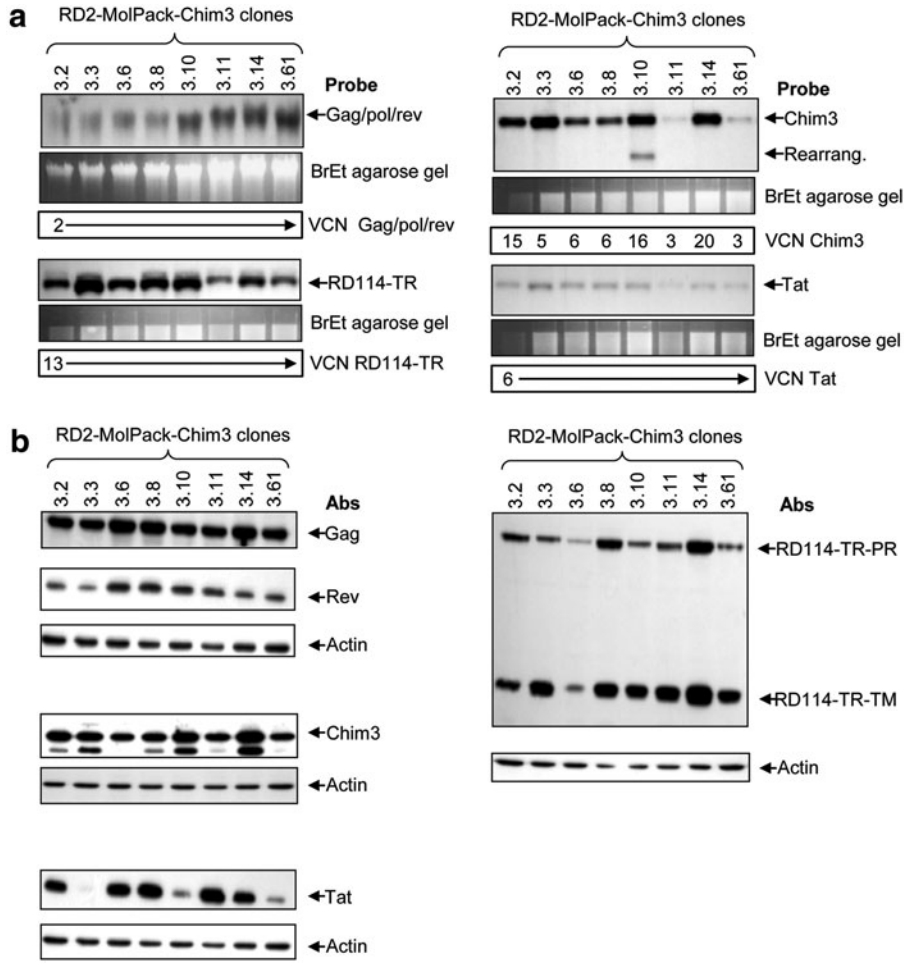

FIG. 3. Characterization of RD2-MolPack-*Chim3* clones. **(a)** Southern blot analysis of the integrity of the individual genes in eight independent RD2-MolPack-*Chim3* clones. About 8 μg/sample of gDNA was digested with the appropriate restriction enzyme to analyze the corresponding integrity of the different integrated cassettes and hybridized with the specific probes as indicated. **(b)** Western blot analysis of cellular extracts derived from the eight RD2-MolPack-*Chim3* clones. About 40 μg/sample of cellular extracts was probed with the specific antibodies as indicated. After stripping, the membranes were hybridized with an antiactin antibody as control of protein loading.

LV (7.3 ng p24Gag) than VSV-G LV (0.73 ng p24Gag) pp is required to obtain similar VCN (0.44 and 0.50, respectively) (Table 4). The percentage of positivity of $CD34^+$-derived cells transduced with RD114-TR pseudovirions is more stable over time than that of $CD34^+$-derived cells transduced with VSV-G pseudovirions.

Tenofovir prevents autotransduction in RD2-MolPack-Chim3 cells

To evaluate whether RD2-MolPack-Chim3 cells can be autotransduced by the LV they themselves produce, we measured the fold of increase of PΔN-*Chim3* VCN over 1 month of culture in four RD2-MolPack-*Chim3* subclones by Q-PCR. After 30 days of continuous culture, we quantified 1.3-, 1.9-, 1.4-, and 1.7-fold increase of VCN relative to that calculated for each subclone at the beginning of their culture (Supplementary Table S10), indicating that RD2-MolPack-*Chim3* cells endure autotransduction during long-term cultivation. Next, we demonstrated that autotransduction is blocked by the Nucleoside Analog Reverse Transcriptase inhibitors tenofovir, which is a drug authorized in clinic for the treatment of HIV infection. First, we established that tenofovir is the most effective, with no toxicity at the most efficacious dose (50 μM), among other NRTI we tested, abacavir and lamivudine, in preventing PΔN-*Chim3* integration in RD2-MolPack cells (Supplementary Fig. S5). Remarkably, we determined that RD2-MolPack-*Chim3* subclones raised in the presence of tenofovir conserve the VCN of the parental clone in both low-VCN (*Chim3.61*-derived, which carries 3.5 PΔN-*Chim3* VCN) and high-VCN (*Chim3.2*-derived, which carries 20 PΔN-*Chim3* VCN) packaging cells (Fig. 4a, b). Finally, we verified that tenofovir is not toxic for the TV integrity by carrying out Southern blot analysis with the representative subclone RD2-MolPack-*Chim3.61,* cultivated in the presence or absence of tenofovir and showing in all samples a correct-in-size 4 kb band (Fig. 4c). Altogether, these data indicate that tenofovir can be safely used for preventing autotransduction in stable LV production.

Discussion

We report a new technological strategy to develop packaging cells for stable production of HIV-based LV. In particular, we developed the RD2-MolPack-*Chim3* producer clone primarily to go forward with the preclinical anti-HIV gene therapy studies based on *Chim3*-LV (Porcellini *et al.,* 2009, 2010), and secondly to pave the way for the future development of a third-generation stable packaging cell line, RD3-MolPack. The feasibility of HSC-based anti-HIV gene therapy has been demonstrated for different therapeutic genes and payloads (Kitchen *et al.,* 2011; Mitsuyasu *et al.,* 2011; Bovolenta *et al.,* 2012). Nevertheless, a frequent downside for the production of LV carrying anti-HIV transgene is the transgene- or

Table 4. Comparison of Transduction Efficiency of RD114-TR Versus VSV-G-Pseudotyped LV on CD34$^+$ and CEM A3.01 Cells

	Target cells[a]*: CD34*$^+$			
LV	*p24Gag (ng)*	*Transd*[b] *5d (%)*	*Transd*[b] *14d (%)*	*VCN*[c] *14d* (n)
RD114-TR-pseudot.[d] (RD2-MolPack-*Chim3*)	73.0	99.25	96.5	16.9
	7.30	91.50	86.5	7.80
	0.73	42.65	40.0	1.05
VSV-G-pseudot.[d] (HEK-293T cells)	73.0	51.50	30.0	0.95
	7.30	27.90	20.5	0.51
	0.73	05.20	02.8	0.06
	Target cells[a]*: CEM A3.01*			
LV	*p24Gag (ng)*	*Transd*[b] *5d (%)*	*Transd*[b] *14d (%)*	*VCN*[c] *14d* (n)
RD114-TR-pseudot.[d] (RD2-MolPack-*Chim3*)	73.0	93.0	85.0	1.60
	7.30	50.0	51.0	0.44
	0.73	12.0	13.0	0.07
VSV-G-pseudot.[d] (HEK-293T cells)	73.0	99.0	99.0	15.7
	7.30	96.0	97.0	2.96
	0.73	49.0	50.0	0.50

[a]One hit of transduction was performed in the presence of retronectin on 4×10^4 CD34$^+$ or CEM A3.01 cells in 96-well plates. Seventy-three ng p24Gag of VSV-G LV, whose titer was calculated on CEM A3.01 cells, corresponds to MOI = 100.
[b]Transduction efficiency was calculated by fluorescence-activated cell sorting analysis of ΔLNGFR expression at the indicated time points. Values for CD34$^+$ cells are the means of two normal donors.
[c]Vector copy number (VCN) was calculated 14 days after transduction by quantitative polymerase chain reaction.
[d]LV were produced from the RD2-MolPack-Chim3 cells (RD114-TR LV) in the presence of 2 m*M* Na-butyrate, and from transfection of HEK-293T cells of second-generation packaging Chim3 TV and VSV-G plasmids (VSV-G LV).

payload-mediated inhibition of LV production itself (i.e., Rev-M10) (Bahner *et al.,* 2007). In this context, we have previously established that the dominant negative Vif protein Chim3 exerts its antiviral action only in the presence of wt HIV Vif (Porcellini *et al.,* 2009, 2010). Here, we strengthen this result showing no impairment of *Chim3*-LV production from any of the RD2-MolPack-*Chim3* clones, thus validating the feasibility of large-scale manufacturing of RD2-MolPack-*Chim3*-derived LV.

RD2-MolPack-*Chim3* has been designed to produce LV for targeting HSC since it carries the RD114-TR envelope, which has been previously (Sandrin *et al.,* 2002; Neff *et al.,* 2004; Di Nunzio *et al.,* 2007) and in this study shown to have a preferential tropism for CD34$^+$ cells compared with VSV-G envelope. As LV titer mainly depends on the type and number of envelope molecules mounted on each LV and on the number of specific envelope receptors present on the cell type used for its calculation, HSCs contain an higher number of RD114-TR receptor than that present, for example, on CEM A3.01 cells. The titer of nonconcentrated RD2-MolPack-*Chim3.25*-derived LV (1.0×10^6 TU/ml on both SupT1 and CD34$^+$ cells) is comparable to that obtained from non-concentrated RD114-PR LV produced by STAR packaging cell line ($\sim5.0\times10^6$ on HT1080 cells) (Ikeda *et al.,* 2003) and even higher to that produced by transient protocols (5.2×10^4 on HT1080 cells) (Kim *et al.,* 2010; Trobridge *et al.,* 2010). These data confirm and extend our previous observations demonstrating an higher fraction of human hematopoietic

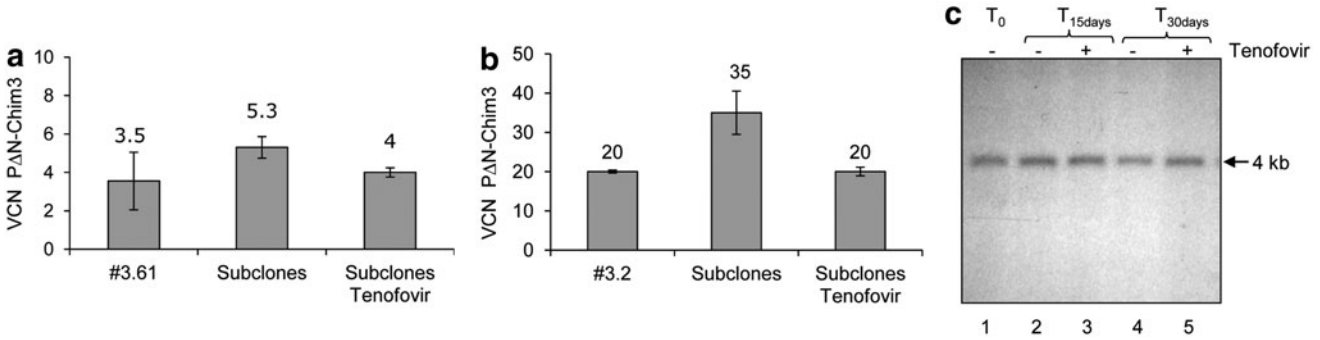

FIG. 4. Tenofovir prevents autotransduction in RD2-MolPack-*Chim3* clones. Quantitative PCR analysis of PΔN-*Chim3* VCN in two RD2-MolPack-*Chim3* clones, the RD2-MolPack-*Chim3.61* clone, carrying 3.5 copies **(a)**, and the RD2-MolPack-*Chim3.2* clone, carrying 20 copies **(b)**, and in several subclones obtained either in the presence or absence of tenofovir. **(c)** Southern blot analysis of gDNA of the representative RD2-MolPack-*Chim3.61* clone, grown either in the presence or in the absence of tenofovir, and digested with *Sac*I to monitor vector integrity over time at three different time points as indicated.

clonogenic progenitors transduced with RD114-TR pseudovirions than those transduced with VSV-G LV (Di Nunzio *et al.,* 2007). These results appear to be at odds with those of Kim *et al.* (2010), reporting an opposite behavior for VSV-G and RD114-TR envelopes in $CD34^+$ cells. We explain this discrepancy by the different experimental conditions applied in the two studies: we used concentrated RD114-TR pseudovirions LV derived from a stable packaging clone instead of LV transiently produced; UCB rather than BM- and mPB; and thereby a different cytokine activation protocol. The lower amount of p24Gag ng/ml (4.7-fold less) produced from RD2-MolPack-*Chim3* cells (mean = 61.2 ± 8.5 SEM ng/ml, $n = 12$) that likely permits the constitutive expression of *gag/pol* genes compared with that produced transiently from HEK-293T cells (mean = 291.8 ± 58.4 SEM ng/ml, $n = 6$) is balanced out by the fact that RD114-TR LV transduce $CD34^+$ cells much more robustly than VSV-G LV do: 100-fold less pp are needed to get equivalent VCN. This implies that the manufacturing scale of RD2-MolPack-*Chim3* supernatant could be ~1/10 respect to the current scale of 25L/lot for the production of VSV-G pseudovirions. Of note, in contrast to VSV-G pseudovirion–transduced stem cells, HSC transduced with RD114-TR LV maintain unaltered expression after 14 days, which likely foresees a better engraftment in patients.

The rationale of integrating the core genes by the Rep78/baculo-AAV hybrid vector system was to hit the AAVS1 region on human chromosome 19, which is the specific and transcriptionally active target for Rep78-mediated AAV integration (Kotin *et al.,* 1990, 1991, 1992; Lamartina *et al.,* 2000). Although the vector landed, instead, into chromosome 2, we obtained results functionally equivalent to those expected for AAVS1 since the 2q32.1 locus is long-term (1 year) transcriptionally active as well. In contrast to other delivery vehicles, for example, plasmid DNA, an integrating vector is expected to persist indefinitely embedded on the host genome. The baculo-AAV vector is tail-to-head oriented, which is the natural configuration of the integrated wild-type AAV and most rAAV vector concatamers (Cheung *et al.,* 1980; Nakai *et al.,* 1999; Musatov *et al.,* 2002). The majority of the vector–vector and vector–cellular genome recombination events occurs within the AAV ITR sequences during concatamerization and integration processes (Nakai *et al.,* 1999; Musatov *et al.,* 2002). Spontaneous deletion and mobilization of the rAAV cassette from the genome integration locus is mediated by rearrangements between the ITRs that define the boundaries of the individual AAV genome subunits (Musatov *et al.,* 2002). Therefore, in PK-7 cells, the documented partial deletion of the 910-bp fragment, central in the concatamer, predicts an unlikely additional event of spontaneous mobilization of the baculo-AAV vector explaining the long-term genetic stability of the packaging cells.

Interestingly, PK-7 clone and RD2-MolPack-*Chim3* constitutively express *gag/pol* and *rev* genes with no cellular toxicity. This is a rare and precious benefit among stable packaging cells because it allows the continuous production of VLP without needing inducers, that is, tetracycline or others, the absence of which must be documented after downstream purification processing.

Furthermore, despite the presence of several homology regions among the viral constructs (i.e., RRE, packaging signal, part of the *gag* gene, and the cPPT element, which is normally contained in the *pol* gene), the sequential strategy we adopted assures biosafety of the system by reducing the risk of recombination events.

Finally, we have ascertained that autotransduction does not interfere with the integrity, functionality, safety, and efficacy of the vectors. We have characterized numerous clones of RD2-MolPack-*Chim3* carrying either high (20) or low (3) copies of the TV *Chim3* without finding correlation between the number of original integrants and the amplitude of the autotransduction process: for both types of packaging cells, the fold of increment of VCN *Chim3* integrants is equal to 1.6. To prevent autotransduction, the use of reverse transcriptase inhibitors such as AZT in previous studies (Vogt *et al.,* 2001; Brandtner *et al.,* 2008) and tenofovir in our study has been proven to be very efficacious.

In conclusion, RD2-MolPack-*Chim3* is the proof of principle that the combination of the Chim3 transgene and the RD114-TR envelope, which does not transduce germline cells (Green *et al.,* 2004), creates a feasible and valid manufacturing tool to be applied potentially *in vivo* (i.e., intrabone delivery) for preclinical studies in animals or clinical applications in humans for HSC-based therapies as anti-HIV gene therapy (Strang *et al.,* 2004; Trobridge *et al.,* 2010).

Acknowledgments

We wish to thank Alessandra Recchia (University of Modena, Italy) for helpful suggestions on AAV-Rep78-mediated integration, and Giuliana Vallanti (MolMed S.p.A.) for helpful suggestions on Q-PCR technology. This work was supported in part by Takara Bio (Otsu, Japan).

Author Disclosure Statement

A.S., B.M.P., S.B., E.Z., S.C., F.S., Cl.B., G.P.R., and Ch.B. are employees of MolMed S.p.A.; F.M. served as consultant to MolMed S.p.A.

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Received for publication September 28, 2012;
accepted after revision June 1, 2013.

Published online: June 14, 2013.