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Lentiviral vectors pseudotyped with murine ecotropic envelope: Increased biosafety and convenience in preclinical research

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Objective. **Lentiviral vectors are increasingly used for preclinical models of gene therapy and other forms of experimental transgenesis. Due to the broad tropism and the ability for concentration by ultracentrifugation, most lentiviral vector preparations are produced using the vesicular stomatitis virus glycoprotein (VSV-g) protein as envelope. Recently, Hanawa and colleagues have demonstrated that the ecotropic envelope protein of murine leukemia viruses allows efficient pseudotyping of HIV-1-derived vector particles. However, this method has found little acceptance, despite potential advantages.**

Materials and Methods. **We produced lentiviral vectors pseudotyped with murine ecotropic envelope using a four-plasmid transient transfection system and evaluated their performance in murine fibroblasts and hematopoietic cells.**

Results. **Titers of lentiviral "ecotropic" supernatants were only slightly lower than those produced with VSV-g, could be concentrated by overnight centrifugation (13,000*g*), and efficiently transduced murine fibroblasts and hematopoietic cells but not human cells. Our Institutional Biosafety Committee agreed on the production and use of replication-defective lentiviral vectors pseudotyped with murine ecotropic envelope under biosafety level 1 (BL1) conditions with additional BL2 practices. We also obtained useful guidelines for the work with human infectious lentiviral vectors.**

Conclusions. **For the researcher, "ecotropic" lentiviral vectors significantly improve the convenience of daily work, compared to the conditions required for lentiviral pseudotypes that are capable of infecting human cells. High efficiency and superior biosafety in combination with convenient handling will certainly boost the potential applicability of this important vector system.** © **2006 International Society for Experimental Hematology. Published by Elsevier Inc.**

Lentiviral vectors based on the human immunodeficiency virus type 1 (HIV-1) and related complex retroviruses are increasingly used for preclinical models of gene therapy and other forms of experimental transgenesis, including oncogene delivery [1,2]. Usually, particles are generated using the safety-enhanced "third-generation" split plasmid system that consists of a plasmid encoding the Rev-dependent genomic vector RNA with self-inactivating long terminal repeats (LTRs), two plasmids expressing the HIV-1 proteins Rev and Gag-pol, and a fourth plasmid encoding the envelope protein of choice [3]. Due to the broad tropism and the ability for concentration by ultracentrifugation, most lentiviral vector preparations are produced using the vesicular stomatitis virus glycoprotein (VSV-g) protein as envelope. However, many other viral glycoproteins can also be suitable for pseudotyping of HIV particles [4,5]. Recently, Hanawa and colleagues have demonstrated that the ecotropic envelope protein of murine leukemia viruses (MLV) allows efficient pseudotyping of HIV-1-derived vector particles, and that concentration of these particles can be achieved by ultrafiltration or ultracentrifugation [5]. However, this method is still not widely used, despite offering significant advantages in preclinical research.

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Materials and methods

Plasmids encoding lentiviral vectors were cloned using standard procedures, resulting in the constructs shown in Figure 1A. All expressed enhanced green fluorescent protein (GFP). The basic construct, pRRL.PPT.PGKGFPpre, was kindly provided by L. Naldini, Milano, Italy. Lentiviral vectors were prepared after calcium phosphate–mediated transfection of subconfluent 293T cells cultured in DMEM, 10% fetal calf serum, 1% sodium pyruvate plus penicillin/streptomycin. For a 10-cm dish, lentiviral vector plasmids (5 μg) were cotransfected with plasmids encoding the HIV-Rev (5 μg) [3], HIV-gag/pol (12 μg) [3] and either the VSV-g glycoprotein (2 μg) [3] or the ecotropic envelope (2 μg) [6]. Supernatants were harvested after 36 and 48 hours and filtrated through 0.22-μm filters. Vector-containing media were aliquoted and stored at −80°C.

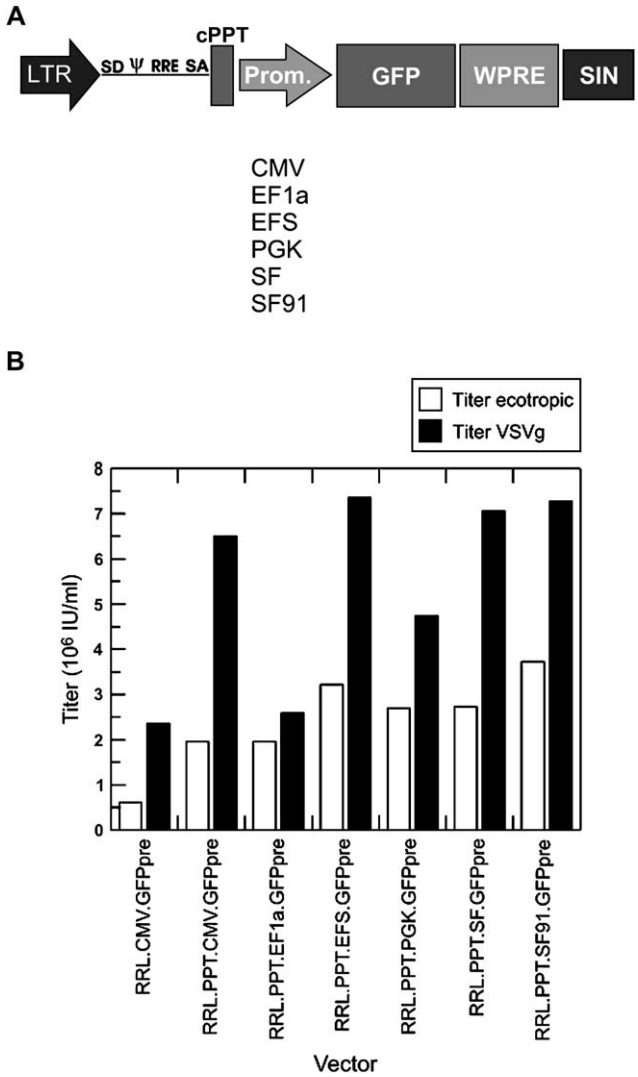

Figure 1. Lentiviral vector production as ecotropic or VSV-g pseudotypes. The vectors used are schematically shown in **(A)**. Third-generation lentiviral vectors (RRL) encoding enhanced green fluorescent protein (GFP) contain the central polypurine tract (cPPT), the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), and internal promoters of the human cytomegalovirus (CMV), the human elongation factor 1 alfa (EF1a), EF1a lacking the first intron (EFS), the human phosphoglycerokinase gene (PGK), the murine spleen focus-forming virus (SF), or SF followed by an intron (SF91). **(B)** Independent of the vector used, ecotropic titers (white columns) were ∼50% (range 25–80%) of VSV-g titers (black columns).

SC-1 cells (murine fibroblasts) were cultured in the same medium. A total of 10^5 cells were plated in a well of a 12-well plate the day before transduction. Thawed viral supernatants were added in the presence of protamine sulfate [4 μg/mL], centrifuged at 400*g* in a cell culture centrifuge for 1 hour, and replaced with fresh medium the following day. EGFP expression was determined by flow cytometry (FACScalibur, Becton-Dickinson, Heidelberg, Germany) 4 days after particle exposure.

Murine lineage-negative bone marrow cells were isolated, cultured, and transduced as described [7]. The multiplicity of infection (MOI) was 2 or 40 (single exposure) using supernatants titrated on SC-1 cells.

To test whether human cells are susceptible to concentrated ecotropic pseudotyped lentiviral vectors, 3×10^5 Jurkat T lymphoblasts were transduced with either VSV-g or ecotropic pseudotyped lentiviral vectors encoding a red fluorescent protein (RRL.PPT.SF.DsRedexp.pre). To allow ecotropic lentiviral particles to infect human Jurkat cells, 3×10^5 cells were transduced with the gammaretroviral vector SF91mCAT1eGFP followed by a second transduction with the ecotropic pseudotyped lentiviral vector RRL.PPT.SF.DsRedexp.pre 13 hours later. SF91-mCAT1eGFP encodes the murine ecotropic receptor fused with enhanced green fluorescent protein (mCAT-EGFP), kindly provided by Lee et al. [8]. Cells were cultured in RPMI, 10% fetal calf serum, and 2 mM L-glutamine. Transduction was assisted by adding 4 μg/mL protamine sulfate and centrifugation for 60 minutes at 400*g* and 32°C.

For PCR analysis genomic DNA was isolated 17 days posttransduction with QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Five hundred ng of DNA were used for PCR amplification of lentiviral DNA sequence using oligonucleotides DsRed1220F (5′-AGCTGGA CATCACCTCCCACAACG-3′) and wPRE(Lenti)R (5′-TGACA GGTGGTGGCAATGCC-3′). Gammaretroviral mCAT1eGFP DNA was detected by using the primers SFfor (5′-CTGCTGCAG CATCGTTCTGTG-3′) and mCAT(AA88)R (5′-ACCAAA CTCGCCGTAGCACAGGCC-3′). PCR was performed by using Taq Polymerase (Qiagen), 2 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 62°C, and 30 seconds at 72°C. As a loading control we amplified chromosomal GAPDH DNA using oligonucleotides GAPDH(Exon1)F (5′-TGAAGGTCG-GAGTCAACGGGTGAGTT-3′) and GAPDH(Intron1)R (5′-TA GGGGAAGGAGGCTCCGGGCAGAT-3′) under identical PCR conditions.

Results

With an expression vector for the murine ecotropic envelope designed by Kitamura and colleagues [6], we were readily able to produce a number of HIV-1-based lentiviral constructs to high titers. To determine the titers achieved with ecotropic pseudotyping, we used 7 different vectors expressing GFP. Depending on the architecture of the vector RNA, we reproducibly obtained up to 3×10^6 infectious

units (IU) per mL of unconcentrated producer cell supernatant (293T cells transfected with the four-plasmid system). Without further improving the murine envelope protein for pseudotyping of HIV vector particles, the titers reproducibly ranged between 25% and 80% of those achieved with VSV-g (Fig. 1). Lentiviral "ecotropic" particles were stable in a single freeze-thaw cycle and could be concentrated (~50×) by overnight centrifugation at 13,000*g* and +4°C (data not shown). Similarly, centrifugal forces might be useful to enhance transduction of target cells [9,10]. When transducing 100,000 murine fibroblasts, a small volume of only 50 μL rethawed unconcentrated supernatant already led to multiple vector integrants per cell (Fig. 2A), as indicated by the increase in the mean fluorescence. Previous experience with dose-escalated gammaretroviral vectors has clearly established the association of multiple copies per cell with increasing levels of transgene expression in uncloned cultures [11,12], and similar results have recently been achieved using lentiviral vectors pseudotyped with VSV-g [13]. In our hands, five supernatants harvested within 72 hours post transfection of cells (plated in a 10-cm dish) gave total yields of up to 10^8 IU, more than sufficient even for larger experiments and dose-escalation studies.

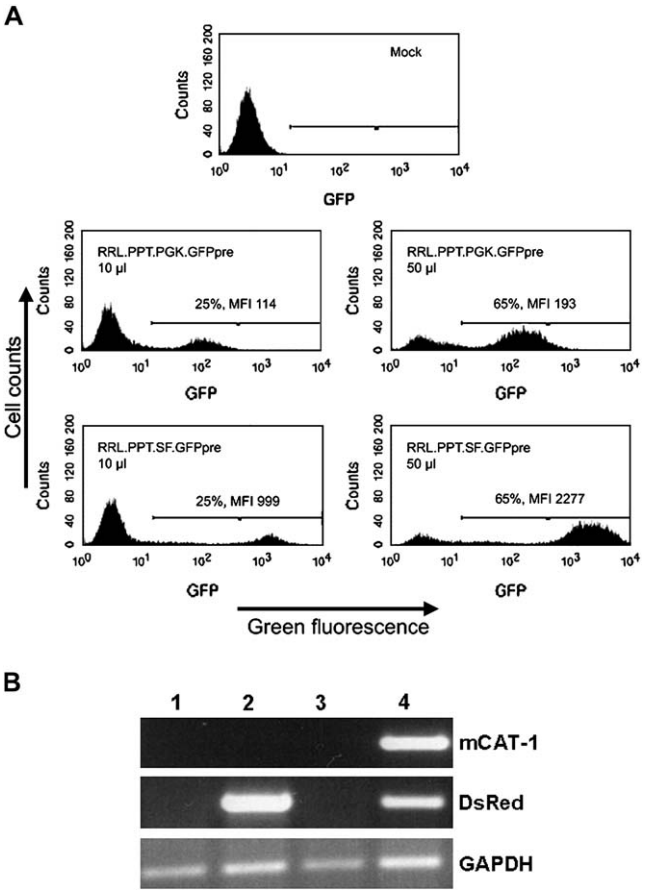

Figure 2. Transduction of murine fibroblasts and inability to transduce human cells. **(A)** Using the ecotropic envelope, 10 μL of unconcentrated tissue culture supernatant were sufficient to transduce 25% of 10^5 murine SC1 fibroblasts. 50 μL already lead to accumulation of multiple vector copies, as indicated by the increased mean fluorescence intensity (MFI) [11]. The MFI obtained at low MOI reflects the strength of the promoters used under conditions of single insertion [12]. **(B)** PCR detecting lentiviral vector sequences in DNA of Jurkat cells, harvested 2 days after exposure to mock supernatant (lane 1), VSV-g pseudotyped particles (lane 2), ecotropic lentiviral particles (lane 3). Lane 4 shows results with DNA of Jurkat cells transduced with the gammaretroviral vector SF91mCAT1eGFP followed by a second transduction with the ecotropic pseudotyped lentiviral vector RRL.PPT.SF.DsRedexp.pre 13 hours later.

To test whether lentiviral particles pseudotyped with the murine ecotropic envelope might transduce human cells, we chose human Jurkat T lymphoblasts that are susceptible to infection with wild-type HIV-1 [14]. As demonstrated by PCR, Jurkat cells were refractory to transduction with "ecotropic" lentiviral vectors, unless cells were modified to express the murine ecotropic receptor (mCAT1) by previous retroviral gene transfer (Fig. 2B). These results were consistent with flow cytometry to detect the DsRed protein encoded by the lentiviral vectors used in these experiments: VSV-g-pseudotyped lentiviral vectors transduced 99.4% of the population, whereas ecotropic lentiviral particles only gave background activity (0.03%). A negative result by PCR suggests that the receptor-mediated block to infection of human cells by "ecotropic" lentiviral particles is tight. If ecotropic lentiviral particles were used to transduce Jurkat cells that were engineered to express mCAT1, at least 75% of mCAT1-positive cells expressed the DsRed vector (FACS plots not shown).

Using a transduction protocol modified for lineage-depleted murine hematopoietic cells [7], we easily reproduced efficient marking by a low MOI of "ecotropic" lentiviral vectors. Increasing the MOI further improved the efficiency of gene transfer (Fig. 3).

Discussion

We found the pseudotyping of HIV-derived vectors with the murine ecotropic envelope most useful for our studies in preclinical mouse models of human gene therapy. The ecotropic envelope mediates efficient transduction of some important cell types such as hematopoietic cells and avoids toxic side effects that can be observed with particle preparations when using VSV-g for pseudotyping [15]. Hanawa et al. previously showed that murine hematopoietic cells transduced with a low MOI (2–4) of "ecotropic" lentiviral vectors achieve efficient long-term multilineage gene marking in transplanted mice [5]. Although not tested here, it is likely that ecotropic lentiviral vectors will also be efficient for gene transfer into murine T cells, because in the context of gammaretroviral vectors the ecotropic envelope has been found more efficient for this population than VSV-g [16]. However, the efficiency for the target population of interest needs to be determined before relying on "ecotropic" particles to replace conventionally used pseudotypes such as

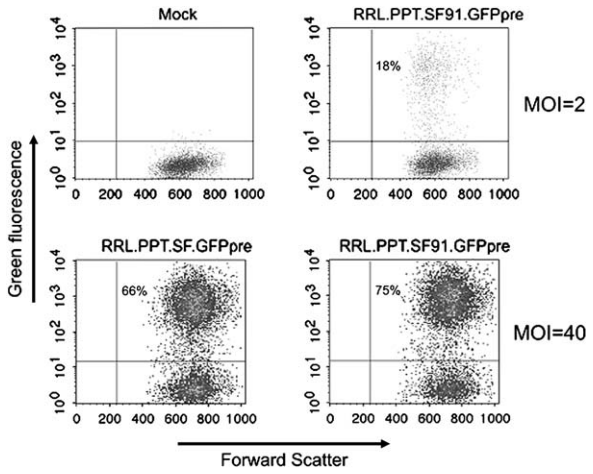

Figure 3. Transduction of murine hematopoietic cells. Lineage marker–depleted cells obtained from steady-state bone marrow of C57Bl/6 mice were cultured for 48 hours in serum-free media supplemented with cytokines stem cell factor, Flt3-ligand, interleukin-11, and interleukin-3 as described [7], followed by a single round of transduction at an MOI of 2 (upper panel) or MOI of 40 (lower panel). Cells were analyzed by flow cytometry for GFP expression 3 days after transduction. Increasing the MOI to 40, ~70% of the cells could be transduced.

VSV-g. Moreover, it should be noted that several questions remain to be addressed, especially in the context of hematopoietic stem cells as targets. These include the potential utility of ecotropic lentiviral vectors to efficiently transduce purified populations of HSCs, under conditions of minimal cytokine stimulation [17,18], and the clonal variability of transgene copy number following "ecotropic" transduction. The stability of transgene expression over time is expected to depend on these parameters, and on the design of the transgene cassette.

A clear advantage of "ecotropic" lentiviral particles is that they cannot transduce human 293T cells used for vector production. This not only avoids the risk of progressive vector instability potentially caused by serial self-infection of packaging cells [19] but also further reduces the risk of generating replication-competent lentivirus. Thus, the murine ecotropic envelope is also of substantial interest for the generation of stable packaging cells, based on the improved technology developed by Takeuchi et al. [20].

It should be noted that the term "ecotropic" indicates that a given retrovirus only infects cells of the species that represents its natural habitat. A more appropriate description of these particles is "lentiviral vectors pseudotyped with murine ecotropic envelope." These rodent-restricted lentiviral vectors should be of particular interest when expressing oncogenes from lentiviral vectors. However, these vectors are only useful for preclinical research and cannot replace the preclinical efficiency and toxicity evaluation of human-infectious particles prepared for clinical studies.

After consulting the Office of Biotechnology Activities (OBA) and members of the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH), the Institutional Biosafety Committee (IBC) of Cincinnati Children's Hospital Medical Center agreed on the production and use of replication-defective lentiviral vectors pseudotyped with murine ecotropic envelope under biosafety level 1 (BL1) conditions with additional BL2 practices as outlined in Appendices G-II-B-2-h (to avoid skin contamination) and G-II-B-2-j (to avoid self-inoculation) of the NIH Guidelines for Research Involving Recombinant DNA Molecules (http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html). In addition, NIH recommended a class 2 biological safety cabinet should be employed for cell culture work. This is largely consistent with rules for ecotropic gammaretroviral vectors such as those based on MLV, and compatible with practice in many European countries, Canada, and Australia (personal communication with F.L. Cosset and M. Cavazzana-Calvo, France; A. Thrasher, Great Britain; V. Prasolov, Russia; M. Verstegen, The Netherlands; R.K. Humphries, Canada; and P. Taylor, Australia).

We have also been in consultation with researchers in various countries and the National Gene Vector Laboratory (NGVL) at Indiana University to benefit from their experience and expertise, in order to develop useful and safe rules for work with lentiviral pseudotypes that are capable of infecting human cells. For these, NIH typically recommends the use of BL2 facilities with BL3 equipment and practices (BL2+). This implies that researchers are asked to demonstrate the absence of replication-competent lentivirus (RCL) before being able to use the vectors under regular BL2 conditions. Of note, regular BL2 rules are sufficient in many European institutions to work with VSV-g pseudotyped lentiviral vectors. NGVL at Indiana University (K. Cornetta, personal communication) has provided information on the safety of the third-generation lentivirus vector packaging systems and advised that in over 4 years of testing for RCL, evidence of productive recombination occurring towards the generation of RCL has not been obtained (although the structure of a putative RCL still has to be defined). The IBC of Cincinnati Children's Hospital has therefore resolved to lower the containment from BL2+ to BL2 for the use of third-generation lentiviral vectors (prepared using a four-plasmid packaging system) with tropism for human cells under the conditions listed in Table 1.

While the biosafety requirements will certainly depend on the vectors, transgenes, and target cells of interest and are subject to specific institutional guidelines, we are strongly encouraged to use lentiviral vectors pseudotyped with murine ecotropic envelope in basic and preclinical research. High efficiency and superior biosafety in combination with convenient handling will boost the potential applicability of lentiviral vectors. Moreover, the recently obtained permission to work under regular biosafety level 2 conditions when using human-infectious third-generation lentiviral vectors as outlined in Table 1 will increase the ease of use of this important vector system.

Table 1. Updated biosafety conditions for using lentiviral vectors of Cincinnati Children's Hospital (January 2006)

	BL1+	BL2	BL2+
Lentiviral vector type*	Third-generation HIV-based	Third-generation HIV-based	First- or second-generation HIV-based
Envelope†	Murine ecotropic	Human-infectious	Human-infectious
Target cells	BL1	BL2	BL2+
RCL testing	Not required for either cell culture or animal work	Not required for either cell culture or animal work	Required for working at regular BL2 conditions
Additional practices	Additional BL2 practices as outlined in Appendices G-II-B-2-h (to avoid skin contamination) and G-II-B-2-j (to avoid self-inoculation) of the NIH Guidelines for Research Involving Recombinant DNA Molecules. NIH also recommended a class 2 biological safety cabinet for cell culture work.	Investigators who wish to use older-generation lentiviral vectors have the option of having the vector certified RCL free (based upon a single PCR analysis) and working at BL2.	RCL testing requirements apply to each individual vector, and once tested subsequent preparations from the same vector are considered BL2.

*According to Ref. 3. RCL, replication-competent lentivirus.

†Depending on the nature of the transgene, ecotropic particles might be classified BL2 and human-infectious third-generation particles BL2+. Features of the target cells represent another independent determinant of the biosafety classification.

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