Efficient Human Hematopoietic Cell Transduction Using RD114- and GALV-Pseudotyped Retroviral Vectors Produced in Suspension and Serum-Free Media

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Abstract

Retroviral vectors derived from the Moloney murine leukemia virus have been used in successful and promising gene therapy clinical trials. However, platforms for their large-scale production must be further developed. As a proof of principle, we reported the generation of a packaging cell line that produces amphotropic retroviral vectors in suspension and serum-free medium (SFM). In the present study, we have constructed and characterized two retroviral packaging cell lines designed for gene transfer in hematopoietic cells. These cell lines grow in suspension and SFM, and produce high-titer RD114- and gibbon ape leukemia virus (GALV)-pseudotyped vectors for a 3-month culture period. Viral particles released are as robust during repeated freeze–thaw cycles and on thermal inactivation at 37°C as their counterparts produced in cells cultured adherently with serum. We also show that RD114- and GALV-pseudotyped vectors produced in suspension and SFM efficiently transduce human lymphocytes and hematopoietic stem cells. As these retroviral packaging cell lines distinctively maintain high vector titers while growing in suspension and SFM, we conclude that these cell lines are uniquely suitable for large-scale clinical-grade vector production for late-phase clinical trials involving gene transfer into hematopoietic cells.

Introduction

THE POTENTIAL of Moloney murine leukemia (MLV)-derived retroviral vectors in gene therapy has been demonstrated by the successful treatment of patients with X-linked severe combined immunodeficiency (SCID-X1) disease and with adenosine deaminase deficiency (Cavazzana-Calvo *et al.*, 2000; Aiuti *et al.*, 2002, 2009; Gaspar *et al.*, 2004). In these clinical trials, harvested autologous hematopoietic stem cells (HSCs) are transduced *in vitro*, and subsequently re-infused into patients. It is admitted that these successes largely rely on the selective advantage of transduced cells conferred by the transgene (Fischer and Cavazzana-Calvo, 2008). However, this property is not universal, and in the majority of diseases that may be treatable by a stem cell-directed gene therapy approach, gene transfer levels achieved would be below therapeutic thresholds. Optimized clinical

protocols and better gene therapy products would be required to increase the number of genetically modified cells. For example, mild myelosuppressive treatment led to an increase in genetically modified cells due to better engraftment of the transduced HSCs (Aiuti *et al.*, 2002; Ott *et al.*, 2006). Also, the use of high-titer retroviral vectors with better transducing abilities would most likely target more primitive HSCs (Brenner *et al.*, 2003; Relander *et al.*, 2005).

MLV-based retroviral vectors are vehicles of choice to genetically modify lymphocytes. One attractive strategy for cancer treatment is to redirect the specificity of T cells toward tumor cells by introducing physiological or chimeric antigen receptors in these cells (Sadelain *et al.*, 2003; Murphy *et al.*, 2005). This approach has shown encouraging results in melanoma patients (Morgan *et al.*, 2006), and it is now under evaluation for treatment of leukemia (Jensen *et al.*, 2007; Brentjens *et al.*, 2008) and various other tumors. A second

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gene therapy strategy currently in a phase 3 clinical trial involves the genetic manipulation of T cells for controlling graft-versus-host disease (GVHD) (Bonini *et al.*, 2007; Ciceri *et al.*, 2007). The graft-versus-leukemia effect of allogeneic T cells exploited to treat hematologic malignancies is often associated with life-threatening GVHD (Appelbaum, 2001). GVHD can be controlled by ganciclovir administration if the allogeneic T cells have been engineered to express the herpes simplex virus thymidine kinase gene (Ciceri *et al.*, 2005). These two approaches could be readily extended to other types of cancers, and therefore, a large amount of clinical-grade retroviral vectors will be required in the near future.

We have started to address the limitations associated with the large-scale production of retroviral vectors by designing a new generation of retroviral packaging cells. We reported the description and characterization of the 293GP-A2 cell line, which produces retroviral vectors pseudotyped with the amphotropic (MLV-A) envelope protein. This packaging cell line can release vectors at titers up to 4×10^7 infectious viral particles (IVP)/ml, but most importantly it possesses the unique ability to grow in suspension and serum-free medium (SFM) (Ghani *et al.*, 2007). Culture in suspension could allow the production of vectors in bioreactors with practically no size limit, and culture in SFM would lead to viral batches with higher safety profiles (Merten, 2004).

It has been suggested that RD114- and gibbon ape leukemia virus (GALV)-pseudotyped retroviral and lentiviral vectors are more efficient to transduce hematopoietic cells than vesicular stomatitis virus glycoprotein (VSV-G)- and MLV-A-pseudotyped vectors (Gallardo *et al.*, 1997; Kiem *et al.*, 1997; Onodera *et al.*, 1998; Sandrin *et al.*, 2002; Brenner *et al.*, 2003; Lucas *et al.*, 2005; Relander *et al.*, 2005). One explanation for these differences could be the variable levels of each specific retroviral receptor expressed on target cells (Orlic *et al.*, 1996; Brenner *et al.*, 2003; Lucas *et al.*, 2005). Also, RD114-pseudotyped retroviruses seem more potent than other pseudotyped vectors when combined with RetroNectin, a commonly used enhancer for the transduction of HSCs (Sandrin *et al.*, 2002).

In this study, we constructed two retroviral packaging cell lines that produce retroviral vectors pseudotyped with RD114 and GALV envelope proteins for gene transfer into hematopoietic cells. These retroviral producer cell lines were adapted to grow in suspension and SFM, and were characterized and compared in terms of growth, viral production, and stability. Vectors harvested from these packaging cell lines were assessed for their efficiency to transduce human peripheral blood lymphocytes (PBLs) and HSCs.

Materials and Methods

Plasmids

Plasmids used to generate the various packaging cell lines were generated as follows: RD114 and GALV envelope genes were amplified by polymerase chain reaction (PCR) from the RD114 Sc3C provirus (a gift from S.J. O'Brien, Laboratory of Genomic Diversity, National Cancer Institute, Frederick, MD) and from extracted DNA of PG13 cells, respectively (Miller *et al.*, 1991). The 1.7-kbp RD114 envelope gene was amplified with forward primer 5'-ccgctcgaatgaaactccaaca-3' and reverse primer 5'-cggaattctaatctgagctt-3' (the *Xho*I and *Eco*RI sites are underlined). The 2.1-kbp GALV enve-

lope gene was amplified with forward primer 5'-gta gaattcgatggattgctgcct-3' and reverse primer 5'-ccgctcgaatgaaactgagctt-3' (*Eco*RI and *Xho*I sites are underlined). The envelope genes were subsequently cloned in the eukaryotic expression plasmid pMD2.iPuro^r (Ghani *et al.*, 2007). The description of GFP3, the MLV transfer vector carrying the gene for green fluorescent protein (GFP), has been previously reported (Qiao *et al.*, 2002).

Packaging cell lines

The cell line 293GP-A2, which releases amphotropic pseudotyped vectors, has been thoroughly described in a previous study (Ghani *et al.*, 2007). 293GP-GLV9 (producing GALV-pseudotyped vectors) and 293GP-R30 (producing RD114-pseudotyped vectors) packaging cell lines were generated in the same manner as 293GP-A2 cells (Ghani *et al.*, 2007). Briefly, RD114 and GALV envelope plasmids were introduced by transfection in GP21C cells, a 293SF-derived clone that expresses MLV Gag-Pol (Ghani *et al.*, 2007). Cells were then selected for 2 weeks with puromycin (0.2 µg/ml), and puromycin-resistant clones were screened for vector production. A GFP3 transfer vector was stably introduced in 293GP-GLV9 and 293GP-R30 clones by infection with VSV-G-pseudotyped recombinant GFP retroviruses, and 293GP-GLV9/GFP and 293GP-R30/GFP cells were sorted by fluorescence-activated cell sorting (FACS) for GFP fluorescence.

Cell culture

TE671 (ATCC CRL-8805; American Type Culture Collection, Manassas, VA), a 293SF-based packaging cell line, was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St-Louis, MO). K-562 (ATCC CCL-243) cells were cultured in RPMI (Invitrogen, Grand Island, NY). These cell lines were maintained in medium supplemented with 10% fetal calf serum (FCS; Bio Cell, Drummondville, PQ, Canada). 293GP-A2/GFP, 293GP-GLV9/GFP, and 293GP-R30/GFP cells were also cultured in suspension and in SFM. The SFM was a low-calcium SFM that was previously described (Ghani *et al.*, 2007). Cells were routinely cultured in 125-ml shaker flasks (Corning Life Sciences, Lowell, MA) at a concentration varying from 3×10^5 to 1×10^6 cells/ml in a 20-ml final volume. They were kept in suspension at a stirrer speed of 120 rpm, 37°C, 100% humidity, and 5% CO₂ (Forma Scientific incubator; Thermo Forma, Marietta, OH) on a Big Bill orbital shaker (Barnstead/ThermoLyne, Dubuque, IA).

Infection and transduction

Virus titers were determined by scoring GFP-positive target cells by FACS analysis. Briefly, TE671 cells were inoculated at a density of 4.5×10^5 cells per well in 6-well plates and cultured in 2 ml of medium overnight. The medium from each well was replaced with 1 ml of serial dilutions of virus supernatants in a 2-ml final volume containing Polybrene (8 µg/ml). Forty-eight hours later, cells were trypsinized and analyzed for GFP fluorescence by FACS. Vector titers were calculated as follows: $\text{titer} = (F \times C_{\text{inf}} / V) \times D$, where F is the percentage of GFP-positive cells, determined by flow cytometry; C_{inf} is the total number of target cells at the time of infection; V is the viral volume applied; and D is the virus dilution factor. Infections resulting in 2–20% of

GFP-positive cells were considered for titer calculation based on the linear range of the assay.

For the transduction assay, K-562 cells were resuspended at 2×10^4 cells per well in a 96-well plate, and incubated with virus at multiplicities of infection (MOIs) of 1, 3, and 10 in 100- μ l final volumes containing Polybrene (8 μ g/ml). GFP fluorescence was analyzed by FACS 2 days after infection.

Transduction of human primary T lymphocytes and CD34⁺ cells

T cells were isolated from human peripheral blood mononuclear cells (PBMCs) collected from healthy donors after obtaining informed consent. After Ficoll-Hypaque (GE Healthcare Bio-Sciences, Baie d'Urfé, PQ, Canada) sedimentation of PBMCs, cells were resuspended in X-VIVO 10 medium (Lonza, Basel, Switzerland) alone, or in X-VIVO 10 medium supplemented with 10% FCS, and incubated in 6-well plates at 1.5×10^7 cells/ml at 37°C for 2 hr. Non-adherent cells were then harvested and activated by beads coated with anti-CD3 and anti-CD28 antibodies for 48 hr in accordance with the manufacturer's instructions (Invitrogen), and cultured in medium supplemented with interleukin (IL)-2 (40 U/ml). Activated lymphocytes at 3×10^5 cells/ml were then added onto 24-well RetroNectin-coated plates (5 μ g/cm²; Takara Bio USA, Madison, WI) that had been previously centrifuged at 1800 $\times$ g with GFP3 retroviruses at 22°C for 2 hr. One day later, half the cells were infected a second time under the same conditions, and GFP expression was measured by FACS analysis 2 days after the last infection.

Frozen purified CD34⁺ cells were activated as previously described (Budak-Alpdogan *et al.*, 2006). Briefly, purified human peripheral CD34⁺ cells were obtained from the Translational Trials Development and Support Laboratory of Cincinnati Children's Hospital Medical Center (Cincinnati, OH) and used as per Memorial Sloan-Kettering Cancer Center (New York, NY) Institutional Review Board approval. CD34⁺ cells were thawed, counted, and cultured at 10^5 cells/ml in X-VIVO 10 medium supplemented with thrombopoietin (TPO, 50 ng/ml), Flt3 ligand (Flt3L, 50 ng/ml), and c-Kit ligand (20 ng/ml). After 24 hr of pre-stimulation, 10^5 CD34⁺ cells in 1 ml of the stimulation medium were transduced with GFP3 retroviruses at MOIs of 1 and 10, using a protocol similar to that used to transduce lymphocytes. Transduced cells were fed with medium supplemented with TPO (50 ng/ml), Flt3L (50 ng/ml), and c-Kit ligand (20 ng/ml) every 2 days. On day 5, cells were harvested for FACS analysis.

Results

Viral titers of GFP vectors produced from 293GP-A2/GFP, 293GP-GLV9/GFP, and 293GP-R30/GFP packaging cell lines

The construction of the 293GP-A2 cell line was reported in a previous study (Ghani *et al.*, 2007), and the engineering of the 293GP-GLV9 and 293GP-R30 retrovirus packaging cell lines is described in Materials and Methods. A transfer vector carrying the GFP gene was introduced by infection in 293GP-GLV9 and 293GP-R30 cells for their characterization. The 293GP-GLV9/GFP and the 293GP-R30/GFP packaging cell

lines were subsequently adapted to grow in suspension and SFM as described for 293GP-A2/GFP cells (Ghani *et al.*, 2007). The impact of the serum-free suspension culture on viral productivity was measured by comparing viral titers before and after adaptation. Viral titers obtained with 293GP-A2/GFP, 293GP-GLV9/GFP and 293GP-R30/GFP producer cells were 4×10^7 , 1.9×10^7 , and 10^7 IVP/ml in adherence with serum, and 4×10^7 , 0.3×10^7 , and 0.5×10^7 IVP/ml in suspension and SFM, respectively (Fig. 1). These results indicate that the adaptation to suspension and SFM did not affect the viral productivity of 293GP-A2/GFP cells, but that it decreased the viral production of 293GP-R30/GFP and the 293GP-GLV9/GFP cells by 2- and 6-fold, respectively.

Growth kinetics and viral production stability of the retrovirus packaging cells cultured in suspension and SFM

A high cellular growth rate is a requirement for the scale-up of eukaryotic cells and, therefore, this parameter was assessed for the packaging cell lines cultured in suspension and SFM (Merten, 2004). Retrovirus producer cells were inoculated in shaker flasks and cultures were monitored during a period of 10 days without changing media. The maximal cell concentrations achieved were relatively similar for the three cell lines: 5×10^6 cells/ml for 293GP-A2/GFP cells, 4.2×10^6 /ml for 293GP-GLV9/GFP cells, and 4.5×10^6 cells/ml for 293GP-R30/GFP cells were obtained. During the exponential growth phase, the viability was more than 95% for all packaging cell lines, and the doubling time measured was approximately 27 hr (Fig. 2a). At the end of the culture period, cell concentration and viability dropped probably as a consequence of nutrient depletion in the media and accumulation of by-products such as lactate (Fig. 2a and data not shown) (Ghani *et al.*, 2006).

FIG. 1. Retroviral production from 293GP-A2/GFP, 293GP-GLV9/GFP, and 293GP-R30/GFP. Vector titers from cells cultured adherently in the presence of serum or in suspension with SFM were measured by limiting dilutions on TE671 cells. Viral supernatants were collected at a concentration of 5×10^6 cells/ml under each condition. Values presented are the means $\pm$ standard deviation of three independent experiments.

FIG. 2. Cellular growth and long-term virus production from packaging cells cultured in suspension and SFM. (a) Growth kinetics of 293GP-A2/GFP, 293GP-GLV9/GFP, and 293GP-R30/GFP cell lines. Cells inoculated at 3×10^5 cells/ml were cultured for 10 days without changing the medium. Cell counts and viability were assessed on each day of the experiment. (b) Long-term vector production stability of 293GP-GLV9/GFP and 293GP-R30/GFP packaging cells. Viral titers were measured from cultured retrovirus packaging cells on days 0, 38, and 94. Values presented indicate the means $\pm$ standard deviation of three independent experiments (a) and one experiment done in triplicate (b).

Another property expected from the packaging cells for large-scale production of retroviral vectors is an ability to sustain stable vector production for a long period of time. The viral production stability of 293GP-A2/GFP cells was previously reported for a 3-month culture period (Ghani *et al.*, 2007). During the same period of time, viral titers were between 1.8×10^6 and 4.9×10^6 IVP/ml without any significant drop for 293GP-R30/GFP and 293GP-GLV9/GFP cells, respectively (Fig. 2b). All retrovirus producer cell lines were then considered stable for long-term viral vector production.

Stability of retroviral particles produced in suspension and SFM

We tested the stability of viral particles as it could be affected by the stringent conditions associated with serum-free suspension culture. First, vector stability during multiple freeze-thaw cycles was compared for all pseudotyped retroviruses produced either adherently with serum or in suspension with SFM. After one cycle, vectors lost between 9.5 and 31% of their original titers. MLV-A-pseudotyped vectors produced in suspension and SFM were less sensitive to freeze-thawing than their counterparts produced adherently in the presence of serum. However, the GALV- and RD114-pseudotyped vectors produced in SFM were slightly more sensitive to freeze-thawing. Increasing freeze-thaw cycle numbers did not necessarily decrease viral activities. A maximal 53% loss in vector activity was observed after three cycles for GALV-pseudotyped vectors produced in suspension and SFM, and for MLV-A-pseudotyped vectors generated from cells cultured adherently with serum (Fig. 3a). The thermal stability of virions was also assessed with the three pseudotyped vectors. Half-lives were relatively similar for viruses in either set of culture conditions: 10–12 hr for the MLV-A- and GALV-pseudotyped viruses, and 6–8 hr for the RD114-pseudotyped virus. Thus, we concluded that viral stability to freeze-thawing and to a temperature of 37°C was not significantly affected by the production of retroviral particles in suspension and SFM as compared with the stability of viruses produced by adherent cells cultured in media with serum.

Transduction efficiency of K-562 cells with the various pseudotyped vectors

The transduction efficiency of retroviral supernatants produced by stable 293GP-A2/GFP, 293GP-GLV9/GFP, and 293GP-R30/GFP packaging cell lines grown in suspension and SFM was tested on the leukemic K-562 cell line. At an MOI of 1, GALV-pseudotyped vectors were the most efficient in transducing cells: 25% versus approximately 15% with the other two pseudotyped viruses. At an MOI of 3, the percentages of GFP⁺ cells increased for the various pseudotyped vectors, but this enhancement was only 4% for MLV-A-pseudotyped vectors. At an MOI of 10, the percentage of GFP⁺ K-562 cells transduced by GALV- and RD114-pseudotyped vectors increased even further to 58 and 59%, respectively (Fig. 4). It is noteworthy that at this MOI, RD114-pseudotyped vectors became as efficient as GALV-pseudotyped vectors. K-562 cells became less infectable at an MOI of 10 as compared with an MOI of 3 with MLV-A-pseudotyped vectors; indeed, the percentage of GFP⁺ cells achieved at an MOI of 10 was 15%, which is similar to the value obtained at an MOI of 1 (Fig. 4). Because a high MOI is often required for primary cell transduction, only viruses produced from 293GP-GLV9/GFP and 293GP-R30/GFP cells were considered for further experiments on primary hematopoietic cells.

Transduction of human peripheral blood lymphocytes with GALV- and RD114-pseudotyped vectors produced in suspension and SFM

As retroviral vectors are vehicles of choice for transducing human PBLs, we next compared GALV- and RD114-

FIG. 3. Stability of retroviral particles produced continuously in suspension and SFM (solid squares) versus adherently with serum (open squares). Viral titers were measured after (a) one, two, and three freeze-thaw cycles of vectors at -80°C and 37°C , and (b) at several time points after vectors were incubated at 37°C . The values presented indicate the means $\pm$ standard deviation of three independent experiments, and they are displayed as a percentage of the initial titer.

pseudotyped viruses for their capacity to transduce these primary cells. PBLs were activated and cultured either in the absence or presence of serum. All experiments were performed in the presence of RetroNectin, at an MOI of 10, and with one or two infection cycles. The percentage of GFP⁺ cells was higher after the second infection cycle for RD114-

pseudotyped vectors: in presence of serum the transduction efficiency increased from 71.8 to 80.6%, and in the absence of serum the percentage of GFP⁺ cells increased from 56 to 71.6% (Fig. 5a and b). The percentage of transduced T cells did not significantly increase after a second round of infection with GALV-pseudotyped vectors: in the presence of

FIG. 4. Transduction of leukemic K-562 cells with vectors produced in suspension and SFM from 293GP-A2/GFP, 293GP-GLV9/GFP, and 293GP-R30/GFP cells. K-562 cells were infected at MOIs of 1, 3, and 10, and 2 days postinfection the transduction efficiency was measured as the percentage of GFP-fluorescent cells. Values presented indicate the means $\pm$ standard deviation of an experiment done in triplicate.

serum the transduction efficiency varied from 63.7 to 65.9%, and in the absence of serum the percentage of GFP⁺ cells varied from 54.6 to 52.7% (Fig. 5a and b). These results indicate that under these experimental conditions GALV- and RD114-pseudotyped vectors transduce T cells similarly after one infection cycle, and that a substantial increase in transduction with a second infection cycle was observed only with RD114 vectors.

Gene transfer into human HSCs with GALV- and RD114-pseudotyped vectors produced in suspension and SFM

MLV-derived vectors are highly efficient in genetically modifying HSCs. GALV- and RD114-pseudotyped vectors produced in suspension and SFM were therefore tested for their ability to transduce HSCs. Human peripheral CD34⁺ cells were prestimulated overnight in serum-free medium and were transduced for 16 hr with MLV vectors. Vector stocks were used at MOIs of 1 and 10 with one or two infection cycles, and 5 days posttransduction GFP expression was measured by flow cytometry. The transduction efficiency increased from an MOI of 1 to 10, and reached 24.7 and 31.6% for RD114-pseudotyped vector after one and two infection cycles, respectively. The percentages of GFP⁺ cells at an MOI of 10 were 15 and 20.9% with the GALV-pseudotyped vector at one and two infection cycles, respectively (Fig. 6). It is noteworthy that the transduction efficiencies obtained at an MOI of 1 with RD114-pseudotyped vectors were on the same order as those achieved with GALV-pseudotyped vectors: 10.1 and 8.5% for one infection cycle, and 10.1 and 11.4% for two infection cycles (Fig. 6). There was no toxicity associated with the transduction of CD34⁺ cells as assessed in a colony formation assay in methylcellulose. The number of colonies and the ratio of erythroid to myeloid colonies were similar for transduced and untransduced CD34⁺ cell samples (data not shown).

Discussion

Although gene therapy has always held promise, it started to show efficacy only in 2000, in the treatment of patients with SCID-X1 (Cavazzana-Calvo *et al.*, 2000). Gene therapy is now in an era of growing clinical impact (Bonini *et al.*, 2007), and with new challenges to be overcome. Among those

FIG. 5. Transduction of human lymphocytes with vectors produced in suspension and SFM from 293GP-GLV9/GFP and 293GP-R30/GFP cells. Activated lymphocytes cultured in the presence (a) or absence (b) of serum were infected at an MOI of 10, and the transduction efficiency was measured as the percentage of GFP-fluorescent cells. The results shown are representative of three independent experiments. 1 $\times$ and 2 $\times$, one and two infection cycles, respectively.

FIG. 6. Transduction of human CD34⁺ cells with vectors produced in suspension and SFM from 293GP-GLV9/GFP and 293GP-R30/GFP cells. Activated human CD34⁺ cells were infected at MOIs of 1 and 10, and the transduction efficiency was measured as the percentage of cells that were GFP fluorescent. 1× and 2×, one and two infection cycles, respectively. The results shown are representative of two independent experiments.

challenges, large clinical batch vectors need to be manufactured for late-phase clinical trials and gene therapy approaches that could become standard therapies (Merten, 2004).

We and others have already started to explore new ways of producing retroviral and lentiviral vectors that would enable the large-scale production of vectors with increased safety profiles (Ghani *et al.*, 2006, 2007; Broussau *et al.*, 2008). We reported as a proof of principle the generation of a high-titer packaging cell line that releases MLV-A-pseudotyped viruses, and that has the ability to grow in suspension and SFM (Ghani *et al.*, 2007). In this study, we now describe the construction and characterization of two packaging cell lines producing GALV- and RD114-pseudotyped viruses in suspension and SFM, and specifically designed for hematopoietic gene therapy. We show that vectors produced from these two cell lines cultured under these conditions were highly efficient in transferring genes into human PBLs and HSCs.

The adaptation of cells to suspension and SFM did not influence the titer of amphotropic viruses, but it led to a titer decrease for GALV and RD114 vectors. The drop in titer was only 2-fold for RD114 vectors, but it was 6-fold for GALV vectors. Nevertheless, titers obtained in suspension and SFM were still greater than 10⁶ IVP/ml with these two pseudotypes (Fig. 1). We have measured the GFP mean fluorescence intensity before and after adaptation to suspension and SFM, and we found that the mean fluorescence was decreased by 4.5-fold for 293GP-GLV9 cells. There was only a 2.5-fold decrease in the mean fluorescence for the two other cell lines (data not shown). This result suggests that expression of the transfer vector is limiting, and that the adaptation to suspension and SFM could lead to a lower expression level of

the transfer vector that could affect viral titers. The retrovirus-packaging cell lines were bulk populations for the GFP3 transfer vector, and we expect to obtain higher titers with the selection of cellular clones for the retroviral genome.

We believe also that the best strategy would be to adapt the parental packaging cell lines to suspension and SFM, and to set up experimental conditions to introduce the retroviral genome by infection or transfection in cells already cultured in suspension and SFM. The scaling up of retroviral vectors with these packaging cell lines would be performed with different types of production systems (stirred tank or wave bioreactors). The continuous feeding of cells by perfusion and vector harvest should also enhance the performance of these packaging cell lines (Merten, 2004; Ghani *et al.*, 2006).

As previously observed with the amphotropic packaging cell line (Ghani *et al.*, 2007), both GALV and RD114 vector productions were stable for at least a 3-month culture period (Fig. 2b). This property is critical because long-term cultures of these retrovirus packaging cell lines in suspension and SFM will be a great asset for future large-scale vector production.

Viral particles produced in suspension and SFM are as stable at 37°C as their counterpart produced adherently in the presence of serum (Fig. 3b). These results are consistent with the data published for lentiviral vectors in which cells were cultured in the presence of serum but with only the production phase performed in SFM (Opti-MEM) (Strang *et al.*, 2004). In the same study, the investigators also addressed the stability of viral particles when freeze-thawed and found that only the VSV-G-pseudotyped vectors were sensitive to the SFM (Strang *et al.*, 2004).

Contrary to RD114- and GALV-pseudotyped vectors, the transduction efficiency of K-562 cells with amphotropic vectors was similar at MOIs of 1 and 10 (Fig. 4). This result can be explained by the presence of virus-free envelopes or “empty” viral particles that can compete for receptor binding with recombinant retroviruses. Similar observations were reported for the amphotropic packaging cell lines FLYA13 and PA317 (Arai *et al.*, 1999; Seppen *et al.*, 2000; Slingsby *et al.*, 2000). The 293GP-A2 packaging cell line produces GFP vectors at higher titers than the 293GP-GLV9 and 293GP-R30 cells, and it could still be useful for applications that do not require the transduction of cells at high MOIs. Furthermore, viral infectivity is likely to be much less affected under conditions in which target cells express a high level of viral receptors or if the vector is delivered *in vivo*. The elimination of potential inhibitors by purification could also be a valuable option to generate vector stocks with good transduction abilities (Segura *et al.*, 2006).

Our results clearly demonstrated the potency of retroviruses produced from 293GP-GLV9/GFP and 293GP-R30/GFP to infect PBLs. Up to 71 and 63% transduction was achieved with only one round of infection with RD114 and GALV-pseudotyped vectors, respectively. After a second infection cycle, the percentage of GFP⁺ cells had substantially increased with the RD114-pseudotyped vector, but was unchanged with GALV-pseudotyped vectors (Fig. 5a and b). On the other hand, although better transduction efficiency was obtained after a second infection of CD34⁺ cells with both GALV- and RD114-pseudotyped vectors at an MOI of 10, the RD114-pseudotyped vector appeared to be the most efficient.

In conclusion, this study reports the generation of two high-titer retrovirus packaging cell lines that release RD114- and GALV-pseudotyped vectors. These cells have the ability to grow in suspension and SFM, and therefore the potential to produce large clinical-grade vector stocks for gene therapy applications that involve the manipulation of hematopoietic cells.

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Author Disclosure Statement

K.G. and M.C. are inventors on a patent that has been licensed to BioVec Pharma. K.G. and M.C. own equity in BioVec Pharma.

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