Original Research

RD114 envelope proteins provide an effective and versatile approach to pseudotype lentiviral vectors

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Abstract

Lentiviral vectors derived from the HIV-1 genome offer great promise for gene therapy due to their ability to transduce non-dividing cells and sustain long-term expression of transgenes. The majority of current lentiviral vectors are pseudotyped with the vesicular stomatitis viral envelope (VSV-G). VSV-G equips lentiviral vectors with a broad host cell tropism and increased stability. Increased particle stability enables viral supernatants to be concentrated by high-speed centrifugation to enhance their infectivity. Despite its efficacy, VSV-G is cytotoxic – a feature that prohibits the development of stable cell lines that constitutively express this envelope. Therefore, non-toxic envelope proteins are being investigated. RD114 is an attractive alternative because it also provides increased particle stability and its receptor is widely expressed on hematopoietic stem cells (HSCs). In this study, the packaging efficiency of three envelope proteins, RD114, RDpro and VSV-G, were evaluated with two lentiviral vectors (TRIP GFP and HPV-402). RDpro is an RD114-HIV chimera designed to pseudotype lentiviral vectors more efficiently. In transient systems, VSV-G generated titers of 10^8 and 10^7 viral particles/mL for TRIP GFP and HPV-402. RDpro possessed titers of 10^7 and 10^6, while RD114 titers were one log lower for each vector. Despite having relatively lower titers, RD114 proteins are less toxic; this was demonstrated in the extension of transient transfection reactions from 48 to 96 h. VSV-G transfections are generally limited to 48 h. In regard to gene therapy applications, we show that RDpro supernatants efficiently transduce peripheral blood HSCs. The versatility of RD114 envelopes was again demonstrated by using a 'mixed' expression system; composed of stably expressed RD114 envelope proteins to pseudotype lentiviral vectors generated in *trans* (titer range 10^3–10^5). Our data show that RD114 envelope proteins are effective and versatile constructs that could prove to be essential components of therapeutic lentiviral gene transfer systems.

Keywords: RD114, envelope proteins, lentiviral vectors, gene delivery, gene therapy

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Introduction

Lentiviral gene transfer systems have been investigated extensively as a method to deliver genes to a variety of cell types such as hematopoietic stem cells (HSCs),[1,2] neurons[3–5] and endothelial cells.[6] Lentiviral gene delivery systems possess two features that the more well-established oncoretroviral systems do not: (1) the ability to integrate into the genome of non-dividing cells; and (2) a greater cloning/packaging capacity that enables the expression of large RNA transcripts without aberrant splicing or degradation.[3,4,7–9] The majority of lentiviral vectors are pseudotyped, or 'packaged', with the envelope glycoprotein from the vesicular stomatitis virus (VSV-G). VSV-G recognizes a ubiquitous phospholipid as its receptor and provides lentiviral vectors with a broad host cell tropism.[10] VSV-G is also very stable. The stability provided by this envelope enables supernatants to be concentrated by high-speed centrifugation to increase their infectivity.[11] Based on these attributes, a great deal of effort has been put forth to develop stable lentiviral cell lines containing VSV-G. Stable cell lines are desirable because they are more amenable to large batch production and have less batch-to-batch variability than transiently expressed viral vectors. Moreover, stable cell lines can be exhaustively analyzed with a single set of safety assays to detect deleterious replication-competent lentiviral particles.[12]

Progress toward the development of stable lentiviral cell lines has been severely limited by the cytotoxicity of VSV-G and portions of the HIV-1 genome (i.e. Pro, Gag and Tat).[11,13–17] Despite early setbacks, several inducible lentiviral cell lines are now available. The majority of these cell lines express VSV-G under the control of tetracycline.[18–23] The most efficient of these systems have titer values that reach 10^7 viral particles/mL (vp/mL).[21,22] Despite their enormous potential, these cell lines are susceptible to genomic instability that results in 'leaky' expression of VSV-G.[21,24] Therefore, non-toxic envelope proteins from alternate viral systems such as murine leukemia virus (MLV), gibbon ape leukemia virus (GALV) and the endogenous feline virus (RD114) are being investigated.[25–29] Pseudotyping lentiviral vectors with alternate envelope proteins has proven to be a difficult process. HIV and simian immunodeficiency virus (SIV) vectors are not packaged effectively with either GALV or RD114 envelope proteins. Each envelope contains refractory residues within their cytoplasmic tail region that limits their cleavage by lentiviral protease enzymes.[25,30,31] The lack of protease compatibility restricts the packaging of lentiviral vectors with GALV and significantly reduces the lentiviral packaging efficiency of RD114. The MLV envelope, however, packages both onco- and lentiviral vectors very efficiently.[25] Sequence homology and site-directed mutagenesis studies show that residues within the C-terminus of MLV are responsible for its enhanced cleavage by retroviral proteases.[25] Attempts have been made to improve the lentiviral packaging efficiency of GALV and RD114. Chimeras of each envelope have been constructed by replacing the tail region of GALV and RD114 with the corresponding region from MLV. The resulting chimeras, GALV-TR and RD114-TR, have been shown to pseudotype lentiviral vectors with increased efficiency.[25,28,30,32]

We believe that non-toxic envelope proteins such as RD114 will become essential components of therapeutic lentiviral vector systems to treat hemoglobin disorders. The RD114 envelope protein is of particular interest because its receptor, the neutral amino acid transporter (RDR), is widely expressed on human HSCs.[33–35] Like VSV-G, RD114 is very stable. Therefore, RD114 supernatants can be concentrated by high-speed centrifugation to increase their infectivity.[26,36,37] This feature was exploited in our previous work; concentrated supernatants derived from a stable RD114 retroviral packaging cell line were used to efficiently transduce cord blood (CB) $CD34^+$ cells.[37] In the current study, the lentiviral packaging efficiency of two RD114 proteins (native and RDpro) was investigated. RDpro is an RD114-HIV chimera that was constructed by replacing the R peptide cleavage sequence of RD114 with the HIV-1 matrix/capsid (MA/CA) cleavage sequence.[28,38] Research has shown that the HIV MA/CA sequence enhances the cleavage interactions between HIV-1 protease and the RDpro envelope during virion formation.[28] The subsequent increase in RDpro cleavage correlates with enhanced titer values for RDpro versus RD114, as shown in our results. The therapeutic potential of RDpro was also investigated by using concentrated supernatants to transduce peripheral blood HSCs ($PBCD34^+$ cells). Since RD114 is non-toxic, this envelope can be used in a wider variety of expression/transfection formats than cytotoxic envelopes. In transient expression systems, the collection period for RD114 supernatants can be extended from 48 to 96 h. Typical transient transfection reactions using VSV-G are limited to 48 h. The versatility of RD114 envelopes is best demonstrated in the 'mixed' expression format. The mixed expression system is composed of stably expressed RD114 clones that package lentiviral vectors generated in *trans*. In this expression format, stable RD114 clones pseudotype lentiviral vectors more efficiently than RDpro clones. Our experiments show that RD114 proteins are versatile constructs that efficiently package lentiviral vectors and should be developed further to become essential components of therapeutic lentiviral vector systems.

Materials and methods

Cell lines and $PBCD34^+$ cells

Human 293-T and HeLa cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cryopreserved human $PBCD34^+$ cells were kindly provided by the University of Washington (Seattle, WA, USA). To determine the percentage of $CD34^+$ and $CD34^+38^-$ cells, cells were thawed in a solution of 1× phosphate-buffered saline/1% FBS, stained with anti-human CD34-PerCP and anti-human CD38-APC (Becton-Dickinson, San Jose, CA, USA) and analyzed with a FACSCalibur (Becton-Dickinson) using Cell Quest software (Becton-Dickinson). All cells were grown at 37°C and 5% CO_2.

Packaging and transfer vector plasmids

A three-plasmid transient transfection system was used to generate lentiviral vector supernatants.[39–41] The plasmids used in the transfection reactions are displayed in Figure 1. The HIV-1 packaging plasmid CMVΔR8.2 was used to provide the structural and accessory proteins of the lentiviral vector particles.[42] The TRIP GFP transfer vector, described previously by Zennou *et al.*,[43,44] expresses GFP driven by the cytomegalovirus (CMV) promoter. The second transfer vector, HPV-402, kindly provided by P Leboulch, is a dual-expression vector that co-expresses GFP driven by the phosphoglycerate kinase promoter and β-globin driven by truncated regions of the β-globin locus control region. In the current study, titers for HPV-402 were based solely upon GFP expression.

Envelope plasmids

Three envelope proteins, VSV-G, RD114 and RDpro, were evaluated to determine the ability of each to pseudotype both lentiviral vectors (Figure 1). The VSV-G plasmid (pMD.g) was kindly provided by Didier Trono.[4] RD114 and RDpro plasmids were kindly provided by F L Cosset. Each RD114 envelope protein was inserted into the

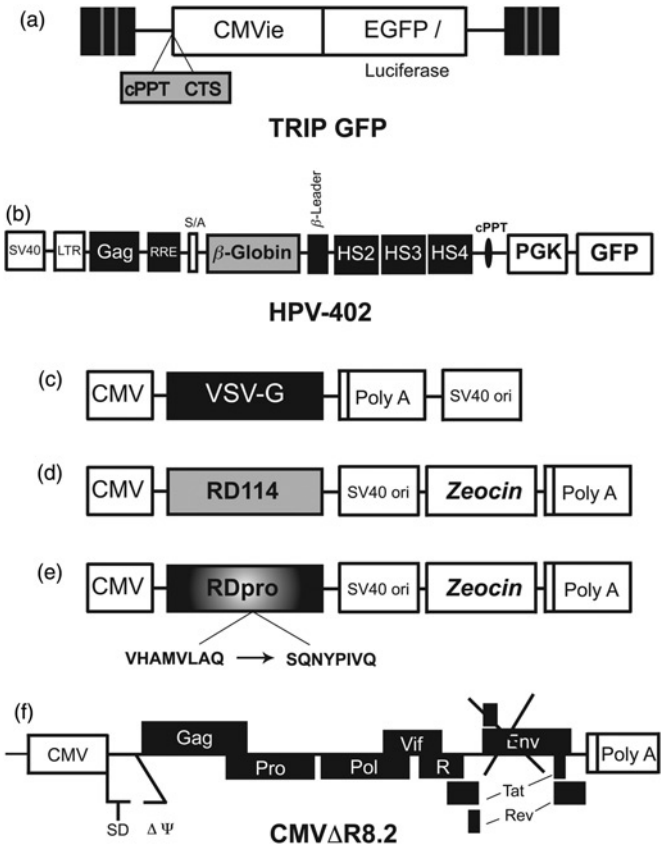

Figure 1 Schematic of viral constructs used in transfection reactions: (a) TRIP GFP enhanced GFP transfer vector, (b) HPV-402 dual GFP/β-globin transfer vector, (c) VSV-G envelope protein, (d) RD114 envelope protein, (e) RDpro chimeric envelope protein and[4] (f) CMVΔR8.2 lentiviral packaging construct. VSV-G, vesicular stomatitis viral envelope

pcDNA3.1/Zeo(+) expression plasmid (Invitrogen, Carlsband, CA, USA).

Production and concentration of lentiviral supernatants

TRIP GFP and HPV-402 supernatants were generated by calcium phosphate transfection of 293-T cells. The transfection reaction was initiated by adding the transfer vector TRIP GFP or HPV-402 (8.0 μg), the packaging plasmid CMVΔR8.2 (12.0 μg) and each envelope plasmid to 293-T cells (4.0×10^6 cells plated onto 10-cm plates) in the presence of calcium phosphate following the manufacturer's protocol (Invitrogen). The VSV-G plasmid was added at a lower amount (1.0 μg) to minimize its cytotoxic effect on the 293-T cells; each RD114 plasmid was added at 4.0 μg. Fresh media (11 mL) was added to each plate 12–16 h after transfection and the viral supernatants were collected at 48 h. Supernatants were filtered through a 0.45 μm filter unit and concentrated by spinning at 100,000*g* in a SW-28 rotor at 4°C for 90 min. Viral pellets were suspended in Isocove's modified Dulbecco's medium (IMDM; Life Technologies) supplemented with 20% bovine serum albumin, insulin and transferrin (BIT) serum substitute (Stem Cell Technologies, Vancouver, BC, Canada). Viral supernatants were pooled and titered on HeLa cells. The level of GFP expression was quantified via fluorescence-activated cell sorting (FACS) analysis to determine the titer of each lentiviral vector using the following calculation: FACS infectious units/mL = [(% GFP+ cells) $\times$ (total number of cells transduced) $\times$ (dilution factor)]/(volume of conc. inoculum). The viral yield of the concentrated supernatants was measured using the following equation: Viral yield = (concentrated titer) (volume of conc. inoculum)/(unconcentrated titer)(total viral volume). In a separate set of experiments designed to monitor the change in viral titer with time, RD114 and RDpro supernatants (TRIP GFP) were collected at 24 h intervals up to 96 h and titered as described previously.

Transduction of human PBCD34$^+$ cells

A total of 1×10^5 PBCD34$^+$ (mixed population of CD34$^+$ and CD34$^+$CD38$^-$ cells) were incubated for 24 h at 37°C on Retronectin-coated well plates (Takara Shuzo Ltd, Otsu, Japan) in IMDM supplemented with 20% BIT serum substitute along with the growth factor cocktail containing: 10 ng/mL thrombopoietin, 10 ng/mL interleukin 6, 100 ng/mL Flt3 ligand (Peprotech, Rocky Hill, NJ, USA), 100 ng/mL stem cell factor (kindly supplied by Amgen, Thousand Oaks, CA, USA) and 10 ng/mL granulocyte-stimulating factor (Immunex, Seattle, WA, USA). After a 24-h preincubation period, aliquots of concentrated viral supernatants of TRIP GFP pseudotyped with either RDpro or VSV-G were added directly to the cells at multiplicity of infections (MOIs) of 10:1 and 20:1. RDpro was selected because this envelope provides sufficient MOIs to infect 10^5 PBCD34$^+$ cells. Negative control (mock) transductions were conducted using PBCD34$^+$ cells under identical conditions in the absence of viral supernatant. The transduction efficiency was determined by measuring the level of GFP expression in the total CD34$^+$ population via FACS analysis.

Psuedotyping transiently expressed lentiviral vectors with stable RD114 envelope proteins

Stable RD114 and RDpro clones were isolated by zeocin selection using the Lipofectamine 2000 transfection kit (Invitrogen). Cells were selected with 100 μg/mL zeocin, in eight 24-well plates for 28 days; resistant clones were trypsinized, re-seeded and expanded. The selection generated 53 RD114 and 36 RDpro clones. Individual clones (10^5 cells) were transferred to 60-mm dishes and grown until confluent. Next, CMVΔR8.2 (4 μg) and TRIP GFP (2 μg) were supplied in *trans* via calcium phosphate transfection. After 48 h, the supernatant from each transfected clone was removed, filtered through a 0.45 μm filter unit and titered as described previously.

Results

Pseudotyping lentiviral vectors with RD114 envelope proteins

RD114 and RDpro were used to package two lentiviral vectors, TRIP GFP and HPV-402. Each vector was also pseudotyped with VSV-G to provide a benchmark for efficiency.

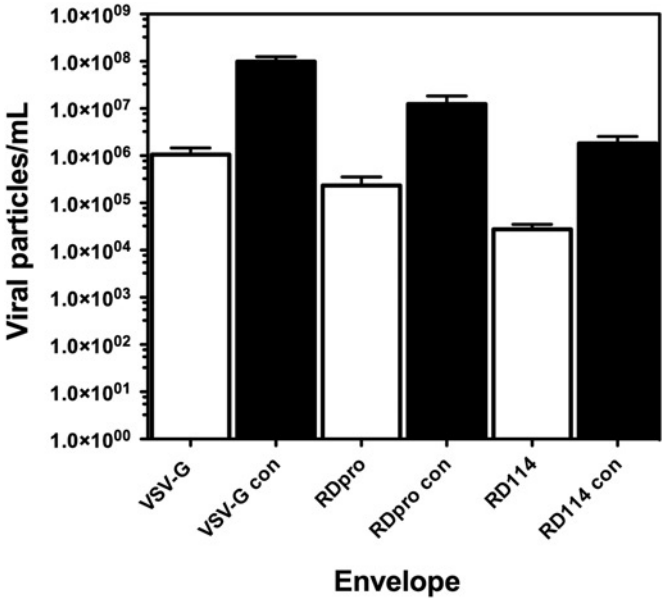

Figure 2 Titer values of the TRIP GFP lentiviral vector pseudotyped with each envelope protein. HeLa cells (2×10^5 cells) were treated with viral supernatants harvested at 48 h. The open bars represent titers from preconcentrated viral supernatants, filled bars correspond to postconcentration titer values. VSV-G, vesicular stomatitis viral envelope

Figure 2 displays the titer values for TRIP GFP with each envelope. Not surprisingly, VSV-G provided the highest titers. The average VSV-G:TRIP GFP titer was 1.10×10^6 vp/mL prior to concentration and increased to 1.0×10^8 vp/mL after concentration. The viral yield of these supernatants was ~46% (Table 1). A direct comparison of the titer values for each RD114 envelope protein revealed that TRIP GFP was more efficiently pseudotyped with RDpro. The average titer for RDpro supernatants was 2.3×10^5 vp/mL prior to concentration and increased to 1.2×10^7 vp/mL postconcentration. The viral yield for RDpro:TRIP GFP supernatants was ~18%. RD114 supernatants possessed the lowest relative titer, with an average value of 2.7×10^4 that increased to 1.8×10^6 vp/mL postconcentration. The viral yield of the RD114 supernatants was ~40%. The titer and viral yield values for each envelope are displayed in Table 1.

Next, the lentiviral packaging efficiency of each envelope with HPV-402 was evaluated. Again VSV-G generated the highest titers; the average unconcentrated titer was 1.2×10^5 and increased to 1.1×10^7 vp/mL postconcentration (Figure 3). The viral yield of VSV-G:HPV-402 supernatants was ~45% (Table 1). A direct comparison of RD114 envelope proteins again revealed that RDpro outperformed RD114. For RDpro, the initial titer of 3.1×10^4 increased to 3.6×10^6 vp/mL following concentration. The viral yield for these supernatants was ~34%. The RD114 titer was 9.1×10^3 prior to concentration and increased to 2.3×10^5 vp/mL postconcentration. In this case, the viral yield was ~17%. The HPV-402 titer values were approximately 1 log unit lower than the TRIP GFP titers, as displayed in Table 1. The lower titers associated with HPV-402 may be a result of the increased complexity of the vector. Each data-set was based on three independent assays. Negative control transfection reactions were conducted using each vector and the packaging plasmid in the absence of envelope proteins. Supernatants collected from the negative control assays did not generate a measurable GFP signal (data not shown).

Monitoring the effect of RD114 supernatant infectivity vs. time

Since RD114 is non-toxic, presumably typical transient transfection reactions using this envelope should generate viable supernatant beyond the standard collection period of 48 h. To investigate this, supernatants were collected at 24-h intervals up to 96 h. Figure 4 displays the TRIP GFP titer profile with each envelope. The titer values for unconcentrated supernatants are denoted with open icons and titers for concentrated supernatants are denoted with filled icons. As shown in Figure 4, the titer for unconcentrated RDpro supernatants declined from 2.3×10^5 to 7.5×10^4 vp/mL. RD114 titers, however, increased from 2.7 to 9.1×10^4 vp/mL during the expression period. The titers for concentrated supernatants displayed a similar profile. The infectivity of RDpro supernatants declined from 1.2×10^7 to 5.0×10^6 vp/mL whereas RD114 titers displayed a slight increase from 1.8 to 4.6×10^6 vp/mL, during the collection period. Each data-set was based on three independent assays.

Transduction of human peripheral blood CD34$^+$ cells

RDpro supernatants were used to transduce PBCD34$^+$ cells in order to investigate the therapeutic potential of this envelope. In previous studies, we show that concentrated RD114 oncoretroviral supernatants efficiently transduced (>50%) cord blood CD34$^+$ (CBCD34$^+$) cells, despite using relatively low MOIs.[37] In the current study, PBCD34$^+$ cells were transduced with lentiviral RDpro supernatants to determine if similar levels of transduction could be reached. RDpro:TRIP GFP supernatants were selected because they provided sufficient MOIs (>5:1) to infect 10^5 PBCD34$^+$ cells. PBCD34$^+$ cells were also transduced with VSV-G supernatants to provide a positive control. Figure 5 displays the level of transduction for each envelope. For RDpro, the transduction efficiency reached a maximum of 40% at an MOI of 20:1. These values reflect an appreciable level of

Table 1 Comparison of titer and viral yield values for TRIP GFP and HPV-402 lentiviral vectors pseudotyped with each envelope protein

Envelope protein	TRIP GFP Titer (vp/mL)		Viral yield (%)	HPV-402 titer (vp/mL)		Viral yield (%)
	Pre-con	Post-con		Pre-con	Post-con	
VSV-G	1.1×10^6	1.0×10^8	46	1.2×10^5	1.1×10^7	45
RDpro	2.3×10^5	1.2×10^7	18	3.1×10^4	3.6×10^6	34
RD114	2.7×10^4	1.8×10^6	40	9.1×10^3	2.3×10^5	17

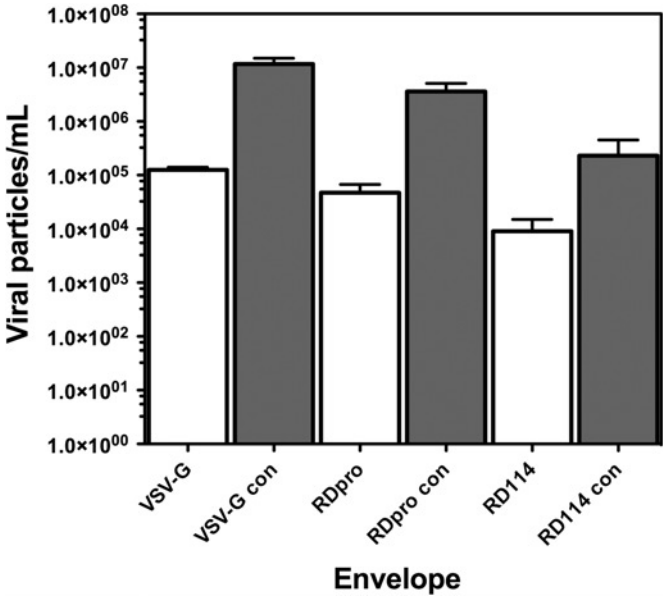

Figure 3 Titer values of the HPV-402 lentiviral vector pseudotyped with each envelope protein. HeLa cells (2×10^5 cells) were treated with viral supernatants harvested at 48 h. The open bars represent titers from preconcentrated viral supernatants; filled bars correspond to postconcentration titer values. VSV-G, vesicular stomatitis viral envelope

transduction but they were slightly lower than the reported PBCD34$^+$ transduction values for RD114-TR.[30,45,46] VSV-G supernatants displayed a transduction maximum of 68% at each MOI. These assays were based on two independent experiments.

Stably expressed RD114 proteins can efficiently package transiently expressed lentiviral vectors

As stated earlier, the development of stable lentiviral cell lines is extremely difficult. Therefore, a more simplified

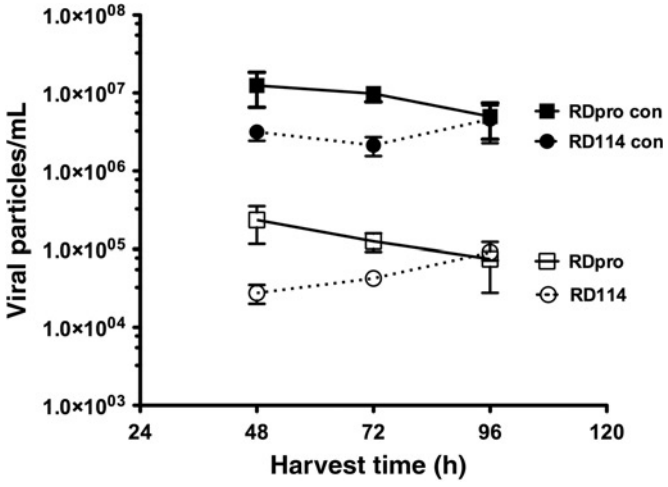

Figure 4 Titer values of the TRIP GFP lentiviral vector pseudotyped with each RD114 envelope protein. HeLa cells (2×10^5 cells) were treated with supernatants harvested at 48, 72 and 96 h. The titer values of supernatants prior to concentration are represented with open icons; the titer values for concentrated supernatants are denoted with filled icons

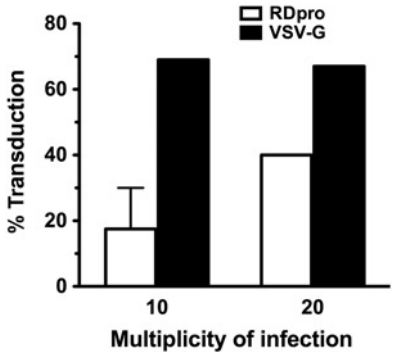

Figure 5 Bar graph representation of the transduction efficiency of PBCD34$^+$ cells (1×10^5 cells) transduced with TRIP GFP lentiviral vectors pseudotyped with RDpro and VSG. PBCD34$^+$ were transduced with supernatants at multiplicity of infection(s) of 10:1 and 20:1. Open bars represent RDpro transduction values; filled bars correspond to VSV-G transduction values. VSV-G, vesicular stomatitis viral envelope

strategy using a 'mixed' or partially stable expression system was developed. Stable RD114 and RDpro clones were isolated via zeocin selection and the lentiviral packaging efficiency of each envelope was evaluated. Thirty-four RD114 and twenty RDpro clones were transferred to 60-mm dishes and allowed to reach confluency. Next, the lentiviral packaging plasmid (CMVΔR8.2) and TRIP GFP were supplied in *trans* via calcium phosphate transfection. Supernatants of each clone were collected and the titer of each was measured as described in the Materials and methods. Surprisingly, RD114 supernatants generated higher titer values than RDpro supernatants. Twelve of 34 RD114 clones generated a measurable titer while two of 20 RDpro clones produced a measurable titer, as shown in Figure 6. The solid bars correspond to RD114 clones and the hatched bars correspond to RDpro clones. The RD114 titer values ranged from 10^3

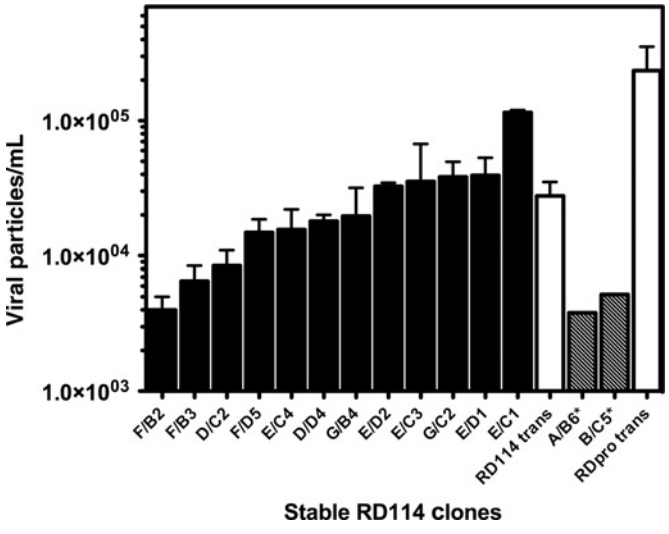

Figure 6 Titer values of 'mixed' lentiviral packaging system. Stable RD114 envelope clones were transiently transfected with TRIP GFP and CMVΔR8.2. Supernatants were collected at 48 h and viral titer was measured with HeLa as described previously. Solid bars represent RD114 titers, hatched bars represent RDpro titer values and open bars correspond to transient titers for each envelope

to 10^5 vp/mL, whereas the RDpro titers were 3.8 and 5.2 × 10^3 vp/mL, respectively. The transient transfection titer values for each envelope were also provided for comparison (open bars, Figure 6). These data were based on three independent assays.

Discussion

Lentiviral transfer of therapeutic genes into HSCs offers great promise, as evidenced by the seminal experiments of Sadelain and co-workers[7] and Leboulch and co-workers[8] to ameliorate β-thalassemia and sickle cell disease in mouse models. The lentiviral vectors used in these studies were generated transiently and pseudotyped with VSV-G. Despite its effectiveness, VSV-G is cytotoxic. Therefore, the development of stable cell lines containing this envelope has been severely limited. RD114 is an attractive alternative because it is non-toxic, stable and its receptor is highly expressed on human HSCs. RD114 is also stable in human sera[30] and resistant to multiple freeze–thaw cycles,[28] factors that provide compatibility for *in vivo* applications and large-scale viral production. We believe these characteristics make RD114 envelope proteins ideal for therapeutic gene delivery systems and provide the basis for our investigations.

A direct comparison of the lentiviral packaging efficiency of RD114, RDpro and VSV-G in transient systems revealed an infectivity profile of VSV-G > RDpro > RD114. The viral yield for each data-set displayed a similar trend, with RD114 envelope proteins displaying more variability (Table 1). The lower viral yield(s) associated with the RD114 proteins may be related to the speed (100,000*g*) used to concentrate the supernatants. Despite their stability, each envelope may have undergone structural damage or dissociation/'shedding' from the virion particle during centrifugation. In the latter case, dissociated or 'soluble' envelope fragments may be concentrated along with intact virions. Hence, the resulting concentrated supernatant contains intact virions and soluble envelope fragments. The latter species may then compete with intact virions for receptor sites on target cells to reduce the overall level of supernatant infectivity. This phenomenon has been shown to occur with the MLV-A envelope.[47,48] Moreover, Strang *et al.*[28] have shown that the viral recovery for RDpro supernatants was enhanced significantly by lowering the centrifugation speed to 10,000 and 3000*g*. Based on these results, it is clear that the optimization of the concentration procedure (i.e. reducing centrifugation speed) will be a critical step to enhance the infectivity and viral yield of RD114 supernatants.

As stated earlier, our goal is to develop therapeutic lentiviral vector systems using RD114-derived envelope proteins. The transduction efficiency of RDpro was evaluated with a relevant therapeutic target, PBCD34$^+$ cells. Previous studies have shown that RD114[37] and RD114-TR[30,45,46] supernatants were capable of transducing CBCD34$^+$ and PBCD34$^+$ cells at moderately high levels. In this study, the maximum level of transduction was 40%. This relatively lower level of transduction may be the result of a cell-specific phenomenon, in that PBCD34$^+$ cells may be less permissive to RD114 supernatants. This view is supported by Di Nunzio *et al.*,[46] who demonstrated that RD114-TR supernatants transduced PBCD34$^+$ cells less efficiently than CBCD34$^+$ and bone marrow (BM) CD34$^+$ cells. In their study, the maximum level of PBCD34$^+$ transduction was 60%. This level of transduction should be attainable for RDpro supernatants that have undergone concentration optimization.

The non-toxic nature of RD114 equips this protein with an added dimension of versatility. This feature was exploited in order to increase the expression period of transient transfection reactions from 48 to 96 h. The extended expression period is ideal for the production of toxic vectors (i.e. HIV and SIV-derived vectors) because it provides a means to potentially increase the total viral yield. Despite having higher initial values the RDpro titers declined gradually, while RD114 titers increased during the expression period. The infectivity/potency trend was consistent for concentrated supernatants for each envelope as well. The decline in RDpro infectivity may be related to the presence of the HIV MA–CA cleavage site. As stated earlier, the insertion of the HIV cleavage sequence increases the interaction between RDpro and the lentiviral protease enzyme during virion assembly. Like VSV-G, HIV-1 protease is cytotoxic.[15–17] Therefore, the elevated level of protease activity (initiated by RDpro) could potentially increase the cytotoxic burden on the 293T producer cells and thus reduce overall viral production. The increase in RD114 supernatant infectivity is a promising result that was somewhat unexpected. Presumably the prolonged expression of toxic lentiviral proteins (Pro, Gag, Pol, Tat) would reduce the fitness of 293-T producer cells – this did not appear to be the case at 96 h. Future experiments that measure the infectivity of supernatants collected at time intervals >96 h are required to determine the limit of 293-T cell viability for RD114 transfections.

To date, there is one stable HIV-1 packaging cell line (STAR cell line).[38] The STAR cell line is composed of a stable retroviral vector that expresses HIV-1 Gag-Pol; this construct is subsequently transfected with an envelope protein to constitute the packaging line. More recently, Throm *et al.*[49] have developed a stable lentiviral cell line that relies on a novel *in vitro* ligation technique to introduce a self-inactivating (SIN) vector to reconstitute the cell line. Both cells lines have shown great promise but the length of time required to generate viral vectors are potential drawbacks. We employed a more basic strategy using stable clones of RD114 and RDpro to package lentiviral vectors generated in *trans*. In this expression format, RD114 clones generated higher titer values than RDpro clones. The titer value of RD114 clone E/C1 was significantly higher than standard transient transfection titers and comparable to transient RDpro titers (Figure 6). These data indicate the enormous potential for RD114 in the mixed expression format and it could prove to be a new method to generate high titer RD114 supernatants. Future experiments must be conducted to: (1) concentrate supernatants produced from the mixed expression system; and (2) use these supernatants to transduce human HSCs. In conclusion, RD114 proteins posses a high level of flexibility and utility and thus offer great promise as components of therapeutic viral vector systems.

Author contributions: All authors participated in the design, interpretation of data and review of the manuscript.

AJB, DF and MW conducted the experiments. AJB wrote the manuscript and AB reviewed the manuscript.

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