Generation of a stable packaging cell line producing high-titer PPT-deleted integration-deficient lentiviral vectors

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ARTICLE
Generation of a stable packaging cell line producing high-titer PPT-deleted integration-deficient lentiviral vectors

Peirong Hu1,2, Yedda Li3, Mark S Sands3,4, Thomas McCown1,5 and Tal Kafri1,2

The risk of insertional mutagenesis inherent to all integrating exogenous expression cassettes was the impetus for the development of various integration-defective lentiviral vector (IDLV) systems. These systems were successfully employed in a plethora of preclinical applications, underscoring their clinical potential. However, current production of IDLVs by transient plasmid transfection is not optimal for large-scale production of clinical grade vectors. Here, we describe the development of the first tetracycline-inducible stable IDLV packaging cell line comprising the D64E integrase mutant and the VSV-G envelope protein. A conditional self-inactivating (cSIN) vector and a novel polypurine tract (PPT)-deleted vector were incorporated into the newly developed stable packaging cell line by transduction and stable transfection, respectively. High-titer (~10^7 infectious units (IU)/ml) cSIN vectors were routinely generated. Furthermore, screening of single-cell clones stably transduced with PPT-deleted vector DNA resulted in the identification of highly efficient producer cell lines generating IDLV titers higher than 10^8 IU/ml, which upon concentration increased to 10^10 IU/ml. IDLVs generated by stable producer lines efficiently transduce CNS tissues of rodents. Overall, the availability of high-titer IDLV lentivirus packaging cell line described here will significantly facilitate IDLV-based basic science research, as well as preclinical and clinical applications.

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INTRODUCTION
The recent successes of integration-competent lentiviral vectors (ICLVs) targeting patients’ hematopoietic stem cells (HSC) in altering the pathologic course of fatal genetic diseases have opened a new avenue in treating incurable diseases.1-4 However, the prospect of broadening the usage of the above therapeutic methodology to include nonfatal diseases is limited by the risk of insertional mutagenesis inherent to all integrating vectors. Indeed, data accumulated from various preclinical studies have raised major concerns regarding the oncogenic potential of gamma-retroviral and lentiviral vectors.5-10 These concerns have materialized in several human clinical trials in which autologous bone marrow transplantation of gamma-retroviral vector-transduced HSC resulted in tumor development.11-12 Taking a similar therapeutic approach, Cavazzana-Calvo et al.13 have successfully employed ICLVs to deliver a β-globin gene expression cassette to patients’ HSC as a means to treat β-thalassemia. However, reports from this trial describe clonal expansion of the myeloid lineage due to vector-mediated insertional mutagenesis, leading to dysregulation of the HMGA2 gene in progenitor cells.

Biosafety concerns regarding ICLV-associated insertional mutagenesis and the desire to harness the advantages of lentiviral vectors—including low immunogenicity, high capacity, and the ability to transduce nondividing cells—were the impetus for the development of integration-defective lentiviral vectors (IDLVs). Generally, IDLVs are packaged into vector particles carrying class I integrase mutants in which one amino acid in the DDE conserved catalytic triad (predominantly the D64 residue) is mutated.14-21 Several studies have demonstrated different levels of illegitimate integrase-independent integration (up to one integration event per 200 vector genomes).14,19,22-24 However, the development of the polypurine tract (PPT)-deleted IDLVs by Kantor et al. has significantly reduced the levels of illegitimate integration.24,25

IDLVs have efficiently transduced a plethora of cell lines and various primary cells including human embryo fibroblasts, human macrophages, HSC, primary neuronal cells, and embryonic stem cells.14,16,18-20,22,24-33 Different laboratories have demonstrated the ability of IDLVs to maintain long-term expression of reporter genes in non-dividing cells in various target organs, including the CNS, spinal cord, muscle, eye, and liver.14,16,18,20,22,24,26-33 Furthermore, Thrasher et al. and Suwanmanee et al. have successfully employed IDLVs in mouse models of gene replacement therapy for degenerative retinal disease and hemophilia B.35,36 The efficacy of IDLVs in cancer immunotherapy and as a means of inducing protective immune responses to human pathogens has been characterized in different experimental settings.37-46 Importantly, a growing number of manuscripts have described IDLVs carrying either zinc-finger nucleases, and the CRISPR-Cas9 system as an effective means of gene editing for clinical and basic science applications.47-52
In the early stages of retroviral vector development, production of gamma-retroviral vectors was premised on stable packaging cell lines. The establishment of the highly transfectable 293T in the early 90’s allowed Pear et al. to produce high-titer retroviral vectors by transient transfection. The ability to pseudotype retroviral vectors with the VSV-G envelope further facilitated production of high-titer retroviral vector particles. Premised on the aforementioned advancements in retroviral vector production, Naldini et al. employed transient three-plasmid transfection to produce and concentrate VSV-G pseudotyped lentiviral vector to transduce murine neurons in vivo. Similar successes employing transient plasmid transfection in producing high-titer lentiviral vectors rendered this approach most popular. Notwithstanding the ease of producing high-titer lentiviral vectors by transient transfection, the need to scale up vector production, to reduce variability between vector preparations, and to reduce the risk of emerging replication competent retroviruses (RCRs) were the drive for the establishment of several stable vector packaging cell lines.

Here, we describe the establishment of the first IDLV packaging cell line, which generates high titer, VSV-G pseudotyped conditional SIN and PPT-deleted IDLVs. The vectors efficiently transduce rodent CNS. The novel packaging cell line will facilitate the scaling up in production of safer IDLVs more suitable for clinical trials.

RESULTS

The establishment of the IDLV inducible stable packaging cell line

Maximizing vector biosafety and titers are the main guidelines to be followed in establishing all lentiviral viral vector stable packaging cell lines. Encouraged by earlier studies of VSV-G pseudotyped lentiviral vectors demonstrating efficient transduction of brain and liver tissues in vivo and HSC ex vivo, we based the first IDLV packaging

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**Figure 1** Schematic outline for generating tetracycline-regulated integration-defective lentiviral vector packaging system. The PVG3 cell line constitutively expresses the synthetic tetracycline-regulated trans activator tTA under the control of a cytomegalovirus promoter, as well as the VSV-G envelope protein and the green fluorescent protein (GFP) marker gene from a bidirectional tetracycline-regulated expression cassette (depicted by a bidirectional arrow). Packaging cell lines were generated by stably transfecting PVG3 cells with the tetracycline-regulated HIV-1 packaging construct pTK1574, followed by G418 selection. p24 enzyme-linked immunosorbent assay was used to identify and screen for high levels of particle production in cell clones expressing minimal — yet detectable — levels of GFP (in the presence of doxycycline (Dox)). Producer cell lines were further established by either transduction or stable transfection.
cell line on this highly effective and broadly used envelope protein. To minimize the likelihood of emerging RCRs, we split the process of incorporating the VSV-G envelope and the packaging expression cassettes into the genome of the future packaging cells to two independent stable transfection procedures (Figure 1). To address the potential hurdle associated with constitutive expression of the cyto-
toxic HIV-1 protease and the VSV-G envelope proteins, the aforementioned expression cassettes were placed under the control of tetracycline-inducible promoters. The IDLV packaging construct (pTK1574) was established by placing an HIV-1 packaging cassette expressing the HIV-1 Gag, Pol, Tat, Rev, and Vpu under the control of a tetracycline inducible promoter (Figure 2a). A D64E HIV-1 integrase mutant encoded by the above Pol gene renders the vector particles generated by the novel inducible packaging cassette integration defective. The efficiency of the novel packaging system at generating IDLVs in a tetracycline regulated fashion was evaluated by transiently transfecting it along with a conventional HIV-1 vector expressing the green fluorescent protein (GFP) under the control of a tetracycline-regulated promoter and the HIV-1 vector pTK945 carrying the GFP marker gene under the control of a cytomegalovirus promoter.53–56 The aforementioned expression cassettes were placed under the control of tetracycline-inducible promoters. 

As a first step in establishing the stable IDLV producing cell line, PVG3 cells were transfected with the inducible IDLV expression cassette (pTK1574, Figures 1 and 2) and a total of 50 G418-resistant cell clones were isolated and screened for minimal, yet detectable GFP expression in the presence of 1 µg/ml Dox. A total of 15 GFP expressing cell clones were identified, and p24 enzyme-linked immunosorbent assay (ELISA) and by scoring GFP expression following serial vector dilutions on 293T cells. In the absence of dox, the novel packaging cassette supports efficient production of physical virions and infectious vector particles (1.42 ± 0.11 x 10^7 IU/ml). Per contra, addition of Dox to culture media effectively inhibited vector production.

Encouraged by these results, we sought to stably transfect the inducible IDLV packaging cassette into the human embryo kidney (HEK) 293-based PVG3 cell line, which was established in an earlier study.58 The PVG3 cells constitutively express the synthetic tetracycline-regulated trans activator tTA under the control of a CMV promoter, as well as the VSV-G envelope protein and the GFP marker gene from a bi-directional tetracycline-regulated expression cassette (Figure 1). As shown in Figure 2b,c, VSV-G and GFP expression is tightly regulated in PVG3 cells. In the presence of Dox, PVG3 cells demonstrate a barely detectable VSV-G and GFP expression. However, Dox withdrawal and addition of sodium butyrate (SB), a broad-spectrum histone deacetylase inhibitor resulted in robust VSV-G and GFP expression (Figure 2b,c).

As a first step in establishing the stable IDLV producing cell line, PVG3 cells were transfected with the inducible IDLV expression cassette (pTK1574, Figures 1 and 2) and a total of 50 G418-resistant cell clones were isolated and screened for minimal, yet detectable GFP expression in the presence of 1 µg/ml Dox. A total of 15 GFP expressing cell clones were identified, and p24 ELISA was used to determine their ability to produce high levels of vector particles

**Figure 2** Generating tetracycline highly regulated integration-defective lentiviral vector (IDLV) packaging cell lines. (a) Depiction of the tetracycline inducible packaging vector pTK1574 containing the gag, pol (encoding the integrase-mutant D64E), vpu, rev, and tat genes under the control of a tetracycline-regulated promoter and the HIV-1 vector pTK945 carrying the GFP marker gene under the control of a cytomegalovirus promoter. (b) Tight regulation of the VSV-G envelope and the IDLV packaging cassettes in PVG3 cells and in the stable packaging cell lines 3–8 and 3–12. The aforementioned cell lines were cultured either in presence of 1 µg/ml Dox or in the absence of Dox and in the presence of 5 mmol/l sodium butyrate. Expression of HIV-1 Gag (p55 and p24), Pol (p66 and p51) and the VSV-G envelope proteins in the above cell lines in the presence and absence of Dox were determined by western blot analysis. (c) Regulation of GFP expression in PVG3 and the packaging cell lines 3–8 and 3–12. The aforementioned cell lines were cultured either in presence of 1 µg/ml Dox or in the absence of Dox and in the presence of 5 mmol/l sodium butyrate. Expression of GFP was determined by fluorescence microscopy. Note that very weak expression of GFP could be detected in the presence of Dox.
Table 1  Stable production of vector particles by packaging cell lines

| Clone name | P24^agg (ng/ml) |
|------------|-----------------|
|            | +Dox           | -Dox day6 | +SB day1 | +SB day2 | +SB day3 | +SB day4 |
| 3–6 Before* | N/A            | 335.78    | 415.40   | 991.79   | 1,286.36 | 1,550.02 |
| 3–8 Before  | N/A            | 358.01    | 1,009.93 | 1,518.69 |          |          |
| 3–10 Before | N/A            | 817.59    | 552.53   | 1,061.29 | 1,493.37 | 1,470.97 |
| 3–12 Before | N/A            | 820.37    | 1,279.10 | 1,769.85 |          |          |
| After       | N/A            | 629.42    | 1,272.87 | 1,282.77 | 1,546.23 |          |
| After       | N/A            | 635.27    | 1,688.21 | 2,079.15 |          |          |
| After       | N/A            | 641.47    | 910.57   | 1,369.99 | 1,361.68 | 1,436.45 |
| After       | N/A            | 628.28    | 1,279.05 |          |          | 1,444.14 |

Table 1  Stable production of vector particles by packaging cell lines

Vector particle production by four packaging cell lines before (a) and after (b) four passages in culture (in the absence of G418) was initiated by Dox withdrawal from culture media. Sodium butyrate (SB, 5mM) was added to culture media after 6 days of induction. After 72 hours in conditioned media, vector particle concentration was determined by p24^agg ELISA. N/A (*) indicates p24^agg measurement not higher than background.

Figure 3  Generating tetracycline-regulated integration-defective lentiviral vector producer cell lines by transduction. (a) Depiction of the cSIN vector pTK136. Note that the 3’U3 region is replaced by tetracycline-regulated element. (b) Vector production by the aforementioned vector-producing cell lines (3-6-136, 3-8-136, 3-10-136, 3-12-136) was initiated by Dox withdrawal from culture media. Sodium butyrate (5 mmol/l) was added to culture media after 6 days of induction. Conditioned media were collected at 2–4 days after addition of sodium butyrate. Viral titers (IU/ml) were determined by scoring GFP expression by fluorescence microscopy following serial dilution of conditioned media on 293T cells.
Vector production was initiated by Dox withdrawal. Sodium butyrate (5 mM) was added to culture media after 6 days of induction. After 72 hours in conditioned media, vector titers (IU/ml) were determined by scoring GFP-positive cells following serial dilution on 293T cells. P24 gag level was determined by ELISA. Numbers represent mean ± SD (n=3). VCNs (viral copy numbers) were measured by qPCR.

above four packaging cell lines (exhibiting the highest p24 gag production) to generate high-titer IDLVs.

To this end, we transduced the four packaging cell lines with the cSIN vector pTK136 (ref. 62) (Figures 1 and 3), from which the GFP reporter gene is expressed under the control of a CMV promoter. Heterogeneous pools of vector-transduced cell lines were induced to generate vector particles by Dox withdrawal and addition of 5 mM SB. Samples of conditioned media were collected between days 2–4 post-SB addition and IDLV titers were determined by scoring GFP positive cells following serial dilution of conditioned media on 293T cells. As shown in Figure 3b, between days 2 and 4 post-SB exposure, the four novel IDLV producer cell lines generated vector titers higher than 10^6 IU/ml. However, IDLV titers (4–5×10^6 IU/ml) generated by cell lines 3-8-136 and 3-12-136 were significantly higher than the titers (1–1.5×10^6 IU/ml) obtained from clones 3-6-136 and 3-10-136. Altogether, these results support the notion that cSIN vectors can rapidly and efficiently establish high titer IDLV producing cell lines.

Establishing the first IDLV PPT-deleted producer cell lines by stable transfection

Although time and labor consuming, stable transfection of plasmid vector DNA into stable packaging cells has been successfully employed to establish stable producer cell lines generating high titer SIN vectors. The fact that the efficiency of stable transfections is independent of the vector's ability to successfully complete its life cycle renders this methodology highly suitable for generating stable producer cell lines of vectors unable to efficiently complete reverse transcription and integration. These include PPT-deleted vectors, which were developed as a means to further enhance vector biosafety by minimizing illegitimate, integrase-independent integration of IDLVs. PPT-deleted vectors cannot efficiently complete reverse-transcription and thus generate minimal levels of linear vector genomes. The majority of PPT-deleted vectors are in the form of 1-LTR circles. Consequently, both integrate-mediated integration of PPT-deleted ICLVs and illegitimate integration of PPT-deleted IDLVs are severely impaired. To combine the advantages conferred by employing a stable vector producing cell line with the enhanced biosafety properties of PPT-deleted vectors, we chose to stably transfect a PPT-deleted vector into stable packaging cell lines. Since both packaging cell lines (3–8 and 3–12) exhibited long-term Dox-dependent expression of GagPol and GFP/VSV-G, and produced high titer cSIN IDLVs (Figures 2 and 3; Table 1), we chose to base the establishment of the first stable PPT-deleted IDLV producer cell lines on the 3–8 and 3–12 packaging cells. To this end, plasmid DNA comprising both the PPT-deleted vector pTK1179 cassette (expressing the GFP marker gene under the control of a CMV promoter) and the Bleocin resistance gene encoding cassette (Figures 1 and 4a) was transfected into the stable packaging cell lines 3–8 and 3–12.

Two heterogeneous 3–8 and 3–12 cell pools and 30 single cell clones were isolated following Bleocin selection. Vector particle production from the above Bleocin resistant cell clones was induced by withdrawal of Dox and addition of 5 mM SB. Concentration of p24 gag and vector titers in conditioned media samples collected at 72 hours post-SB treatment was determined by ELISA and by scoring GFP expression following serial dilutions of conditioned media on 293T cells. As shown in Table 2, both 3–8 and 3–12 cell pools generated comparable levels of p24 gag concentrations and high titers of the PPT-deleted IDLV particles (1.1±0.4×10^6 to 1.6±0.5×10^6 IU/ml). As expected, the above isolated cell clones exhibited a wide range of vector titers, which varied between 1.7±0.5×10^6 IU/ml (clone 3-12-1179-11) to 1.2±0.3×10^6 IU/ml (clone 3-8-1179-1). Note that one cell clone (3-12-1179-14) failed to produce detectable levels of IDLVs (3-12-1179-14). The concentrations of p24 gag in the above conditioned media samples varied between 621±445 IU/ml (clone 3-12-1179-8) and 3,914±1,275ng/ml (clone 3-8-1179-2). Although cell lines producing low vector titers (<10^7 IU/ml) generated p24 gag...
Table 3  Stability of vector producer cell lines

|            | 8–136                  | 12–136                  | 8-1179-1                  | 12-1179-22                |
|------------|-------------------------|-------------------------|---------------------------|---------------------------|
| P24\textsuperscript{ng/ml} | Before\textsuperscript{a} | —                       | 2801 ± 1025              | 2999 ± 109                |
|            | After\textsuperscript{b} | 2374 ± 172              | 1047 ± 70                | 1716 ± 56                 |
| Titer IU/ml | Before                   | 4.0 ± 0.2 \times 10\textsuperscript{6} | 1.2 ± 0.3 \times 10\textsuperscript{7} | 7.5 ± 0.2 \times 10\textsuperscript{7} |
|            | After                    | 5.7 ± 0.5 \times 10\textsuperscript{6} | 5.2 ± 0.4 \times 10\textsuperscript{6} | 7.8 ± 0.3 \times 10\textsuperscript{7} |

Production of integration-defective lentiviral vector’s by four packaging cell lines before (\textsuperscript{a}) and after (\textsuperscript{b}) five passages in culture (in the absence of G418) was initiated by Dox withdrawal from culture media. Sodium butyrate (5 mmol/l) was added to culture media after 6 days of induction. After 72 hours in conditioned media, vector particle concentration was determined by p24\textsuperscript{gag} ELISA. Vector titers (IU/ml) were determined by scoring GFP-positive cells following serial dilution on 293T cells. Numbers represent mean ± SD (n = 3).

Efficient transduction of rodent CNS with stable producing cell line-generated IDLVs

The establishment of a stable IDLV producing cell line was aimed at facilitating large-scale IDLV production for \textit{in vivo} applications. Thus, we sought to characterize the efficacy of IDLVs generated by the above stable vector producing cell lines at transducing rat CNS. To this end, IDLVs generated by cell clones 3-12-136, 3-1179-22 and 3-8-1179-1 were concentrated and purified by ultracentrifugation as described by Kafri \textit{et al.}\textsuperscript{15} Vector titers and concentration of p24\textsuperscript{gag} were determined by scoring GFP expression following serial dilution on 293T cells and by p24\textsuperscript{gag} ELISA. (3-12-1179-22: 2.70 \times 10\textsuperscript{10} IU/ml, p24\textsuperscript{gag} 7.5 \times 10\textsuperscript{7} ng/ml; 3-12-136, 1.28 \times 10\textsuperscript{9} IU/ml, p24\textsuperscript{gag} 6.68 \times 10\textsuperscript{6} ng/ml, respectively). Western blot analysis of concentrated vector particles using primary antibodies to HIV-1 p24\textsuperscript{gag} and reverse-transcriptase proteins, as well as the VSV-G envelope proteins, demonstrated efficient vector particle formation and protein processing, which was comparable to ICLV and IDLV particles generated by transient transfection (Figure 4).

PPT-deleted IDLVs (pTK1179) generated by the novel stable producer cell line 3-8-1179-1 and conventional ICLVs (pTK945) generated by transient transfection were injected into the striatum of 300–350 g male Sprague Dawley rats (total of 0.66 \times 10\textsuperscript{7} IU and 1.2 \times 10\textsuperscript{7} IU respectively). Transduction efficiency was determined at week 5 postinjection by confocal microscopy. As shown in Figure 5, in the rat striatum, ICLVs generated by transient transfection appeared to support moderately greater overall transduction in comparison to the cell line-generated PPT-deleted IDLVs (Figure 5a,b). These findings are in line with the higher number of injected ICLV IU and with an earlier study by Bayer \textit{et al.}\textsuperscript{16} demonstrating reduced

![Figure 4](image-url)
NeuN (Figure 5c,d). Furthermore, in the striatum virtually no GFP—
all of the GFP-positive cells colocalized with the neuronal marker
both ICLVs and IDLVs proved highly neurotrophic, such that almost
ciently transduced murine striatum (data not shown). Interestingly,
observed that IDLV’s generated by a novel producer cell line effi-
transgene expression from episomal lentiviral vectors. Also, we
positive cells exhibited colocalization with glial fibrillary acidic pro-
sum dorsal to the striatum (Figure 5f).

In vivo striatal transduction by integrating and nonintegrating
dentroviral vectors. Five weeks after bilateral 1 µl vector infusions, both the
nonintegrating (panel a) and the integrating (panel b) lentiviral vectors
transduced neurons in the rat striatum. The vast majority of transduced
cells were neurons that colocalized with NeuN (panel c, integration-
competent lentiviral vectors (ICLV); panel d, integration-defective
dentiviral vector (IDLV); NeuN, red). Further example of this neuronal
tropism is illustrated in panel e where confocal microscopy showed
that glial fibrillary acidic protein (GFAP)-positive cells did not colocalize
with GFP for the ICLV (GFAP, red). In contrast, the ICLV, but not the IDLV,
transduced both oligodendrocytes and astrocytes (GFAP, red) in the
corpus callosum dorsal to the striatum (panel f, arrows indicate GFP-
GFAP-positive cells). Panels a,b magnification bar = 250 µm; panels c–f,
magnification bars = 10 µm.

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observed that IDLVs generated by a novel producer cell line effi-
ciently transduced murine striatum (data not shown). Interestingly,
both ICLVs and IDLVs proved highly neurotrophic, such that almost
all of the GFP-positive cells colocalized with the neuronal marker
NeuN (Figure 5c,d). Furthermore, in the striatum virtually no GFP-
positive cells exhibited colocalization with glial fibrillary acidic pro-
tein, a cellular marker of astrocytes (Figure 5e). However, a major
difference between the two vectors was observed in the corpus cal-
lusum dorsal to the striatum. The nonintegrating vector exhibited
little ability to transduce either oligodendrocytes or astrocytes in
the corpus callosum, while the integrating vector efficiently trans-
duced both oligodendrocytes and astrocytes in the corpus callo-
sum dorsal to the striatum (Figure 5f).

DISCUSSION
First stable IDLV packaging
IDLVs offer efficient gene delivery of large genetic cargos into
dividing and nondividing cells with minimal risk of insertional
mutagenesis (inherent in ICLVs). Increasingly, research groups
have been employing these vectors as a means to deliver marker
genesis and therapeutic genetic cargos to a plethora of cell lines
and primary cells in vitro and to various target organs in vivo. The
successful IDLV-based gene replacement therapy in mouse mod-
els of retinal degeneration15 and hemophilia B8 underscore their
clinical potential. Furthermore, IDLVs have been used to transiently
express genome-editing enzymes in dividing cells.69 Indeed, Yi
et al.21 have recently conferred resistance to HIV-1 infection by
mutating the HIV-1 coreceptor CCR5 gene with IDLV-delivered
zinc-finger-nucleases.

To further facilitate the use of IDLV’s, we report here on the
establishment of the first stable IDLV cell lines. Titers of VSV-G-
pseudotyped IDLVs generated by the novel cell lines were higher
than 108 and 1010 IU/ml prior to and after concentration, respect-
ively, and are comparable to or higher than titers of most currently
used IDLVs and ICLVs generated by transient transfection.

Using the tetracycline inducible system, we circumvented the
difficulties associated with overexpressing the cytoxic HIV-1
protease and the VSV-G envelope proteins. Importantly, the stable
packaging cell lines generated in this study exhibited stability and
maintained long-term, Dox-regulated expression of both the VSV-G
envelope and HIV-1 GagPol gene products.

Our strategy for establishing the IDLV producer cell lines focused
on minimizing the likelihood of emerging RCRs. Thus, all expression
cassettes comprising the novel packaging system were introduced
to the host genome separately using several independent rounds
of stable transfection/seletion procedures (Figure 1). Although
time consuming, this approach minimizes the likelihood of
recombination-mediated RCR formation. Recent studies described
the use of gamma-retroviral vectors as a means to incorporate
the tetracycline-regulated trans-activator (TATA), envelope protein
expression cassettes and the HIV-1 packaging system to 293T as
a means to establish stable ICLV packaging cell lines.59,61 Although
efficient, this approach raises biosafety concerns associated with
mobilization of retroviral vectors carrying various components
of the HIV-1 vector producing system, as well as increased likelihood
of recombination-mediated RCR formation. These biosafety concerns
were underscored by a recent publication demonstrating low yet
detectable levels of lentiviral vector packaging of gamma-retroviral
genomes.69 The tight transcriptional regulation of the VSV-G enve-
lope and the HIV-GagPol expression cassettes inhibits ongoing vec-
tor particle formation and consequently prevents superinfection
of the stable packaging cell line with VSV-G-pseudotyped particles.
This further reduces the likelihood of emerging RCRs.

In this study, we employed two different methodologies to intro-
duce the vector cassette into the packaging cell line. In one approach,
we used cSIN vectors generated by transient transfection to trans-
duce the newly developed integrate-deficient packaging cell lines.
This methodology is highly efficient and time saving. A pool of cSIN
vector-transduced packaging cells generated IDLV titers higher than
5 × 108 and 1 × 1010 IU/ml prior to and after concentration. We predict
that screening and isolating highly efficient single cell clones could
further increase vector titers. The cSIN-based approach is optimal
for rapid establishment of stable producer cell lines, yet it is prem-
ised on the ability of vectors generated by transient transfection

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to efficiently integrate into target cell genomes. Thus, it cannot be employed to transfer integration-impaired vectors such as att sites- or PPT- deleted vectors to packaging cell lines. Stable transfection of vector DNA to packaging cells is the second methodology described in this study for generating stable IDLV producing cell lines. We employed this methodology (which is independent of the vector's integration efficiency) to incorporate PPT-deleted vectors into packaging cell lines. Transfection of linearized (nonconcatemerized) vector plasmid DNA followed by Bleo/in selection resulted in the establishment of two stable heterogeneous vector-producing cell pools. These exhibited vector titers higher than 10^7 IU/ml with VCNs of 14 and 59. As expected, 8 out of 30 isolated vector producing cell clones exhibited titers 2–10-fold higher (>10^7 IU/ml) than the titers generated by the above cell pools. The lack of correlation between p24concentration and VCN to vector titers suggests that identification of highly efficient vector producing cell clones cannot be based on these two parameters. The establishment of the first PPT-deleted IDLV producer cell lines enhances the overall biosafety of the IDLV system as it combines the advantage of minimal illegitimate integration conferred by PPT-deleted vectors with the safety and efficiency gained by vector production by stable producer cell lines.

Overall, the establishment of stable PPT-deleted and conventional IDLV producing cell lines facilitates the usage and enhances the safety of the IDLV system, rendering it more suitable for clinical trials.

MATERIALS AND METHODS

Plasmids

The lentiviral vector pTK136, pTK1179, pTK945, and the packaging cassettes ΔNRF (Int-), pTK939 (Int-) were generated, as described previously. The inducible packaging system pTK1574 was derived by cloning a SacII/PvuI cell pools. These exhibited vector titers higher than 10^7 IU/ml with the establishment of two stable heterogeneous vector-producing cell lines. Transfection of linearized (nonconcatemerization of vector DNA to packaging cells is the second methodology transfer assay), as previously described. The emergence of RCRs was ruled out by three independent safety assays (GFP rescue assay, Tat transfer assay, and Gag transfer assay), as previously described.

Titers were determined by scoring GFP expression following serial dilutions on 293T cells. VCN was determined by multiplex PCR on the ABI7300 realtime PCR system. NotI794 primer/probe set (Left primer: 5′-taagaccaggccagaca-3′; Right primer: 5′-cacttcacatggcctca-3′; Roche Universal Probe Library #25, 4686993001, Indianapolis, IN) was used for vectors detection, and paired with human GUSB primer/probe set (Roche 5190525001, Indianapolis, IN) as the reference gene.

HIV-1 p24 ELISA

Titers of physical vector particles were determined by p24 ELISA using the National Institutes of Health p24 Antigen Capture Assay kit, as previously documented.

Western blot

Cells and vector particles were lysed in RIPA buffer and characterized for specific protein content by standard western blot analysis using denaturing 10% SDS–PAGE gels. Blots were probed with a murine anti-VSV-G monoclonal antibody (Ab) (1:1,000; P5D4, Santa Cruz, Dallas, TX), murine anti-HIV-1 RT (1:200, SB282; NIH AIDS Research & Reference Reagent Program, Germantown, MD), and murine anti-HIV-1 p24 Gag (1:5,000, 6457; NIH AIDS Research & Reference Reagent Program) followed by a polyclonal goat anti-murine secondary Ab labeled with horseradish peroxidase (1:10,000; Pierce, Grand Island, NY). Signals were detected by enhanced chemiluminescence (ECL) reagent (GE Health Amersham, Pittsburgh, PA).

Experimental animals

All of the animals were pathogen-free male Sprague–Dawley rats obtained from Charles River. The animals were maintained in a 12-hour light–dark cycle and had free access to food and water. All care and procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. (NIH) 85-23), and all procedures received prior approval by the University of North Carolina Institutional Animal Care and Usage Committee.

For virus vector infusions, rats first were anesthetized with 50 mg/kg pentobarbital and placed into a stereotactic frame. Subsequently, each rat received a bilateral, striatal infusion (1 µl/side) of either the integrating (N = 3) or the nonintegrating (N = 3) lentiviral vector using a 32-gauge stainless steel injector and a Sage infusion pump (interaural line (IAL) 10.0 mm, lateral 3.0 mm, vertical 5.5 mm, according to the atlas of Paxinos and Watson, 1985). The 1 µl infusion occurred over a period of 5 minutes, and the injector was left in place for 3 minutes postinfusion to allow diffusion.

Immunohistochemistry

Five weeks after the vector infusions, the rats received an overdose of pentobarbital (100 mg/kg pentobarbital, i.p.) and subsequently were perfused transcardially with ice-cold 0.1 M sodium PBS (pH = 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4). After overnight fixation in the paraformaldehyde-phosphate buffer, vibratome sections (40 µm thick) were taken and rinsed in PBS. Tissue sections were incubated in 10% normal goat serum and 0.1% Triton X-100 in PBS for 30 minutes. Next, sections were incubated with a primary antibody to NeuN (1:1,000, Chemicon, Pittsburgh, PA) or gial fibrillary acidic protein (1:4,000, DAKO A/S, Glostrup, Denmark) overnight in 3% normal goat serum, 0.2% Triton X-100 and PBS. Tissue sections were then rinsed in PBS, incubated in blocking serum (10% normal goat serum, 0.1% Triton X-100, PBS) for 1 hour and then incubated with a secondary fluorescent antibody (Alexa-fluor 594 goat anti-rabbit, or goat anti-mouse, Molecular Probes) for 1 hour at 4 °C. Following three rinses in PBS, the sections were mounted on slides and coverslipped with fluorescent mounting media. The eGFP and Alexa-fluor 594 fluorescence were visualized on either an Olympus IX 70 fluorescence microscope or a Leica SP2 laser scanning confocal microscope. All cases of colocalization were determined using the confocal microscope and the presence of colocalization within a Z-stack.

CONFLICT OF INTEREST

The technology of the PPT-deleted vector was licensed by UNC to commercial entities and provides royalties to UNC and T.K.

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