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From Lentiviruses to Lentivirus Vectors

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1. Introduction

Although a member of the lentivirus group, the equine infectious anemia virus (EIAV) was the first nonplant virus discovered in the first decade of the 20th century ***(1)***, lentiviruses were considered as rather mysterious viruses until the isolation of the human immunodeficiency virus type 1 (HIV-1) occurred at the beginning of 1980s. Lentiviruses are enveloped viruses carrying two copies of single-strand positive (i.e., codifying) RNA and are considered the ethiologic agents of acquired immunodeficiency syndromes for a broad range of animal species, such as humans, primates, cats, horses, sheep, and goats. Such syndromes develop in multiorgan diseases and share a long period of incubation (with viral persistence despite a potent immunological response) and a fatal outcome. The name lentiviruses (from Latin, *lenti*, slow) originated from the uniquely prolonged incubation period (i.e., from months to years) needed for the infecting virus to induce the disease, a feature joining the most popular lentivirus, HIV-1, with a large number of nonprimates lentiviruses. Lentiviruses belong to the *Lentiviridae* subfamily of the *Retroviridae* family, which also includes the *Oncoviridae*, for the most part viruses inducing cell transformation, and the *Spumaviridae*, viruses establishing persistent as well as nonpathogenic infections (a deeper treatment of this topic can be found in **ref.** ***2***).

Considering that the mode of action of lentivirus vectors is tightly related to the biology of parental lentiviruses, it should be of some utility to gain familiarity with the structure of prototypic members of this viral genus. Introductory remarks will be referred mainly to HIV-1, which shows only minimal structural differences with respect to the other members of the

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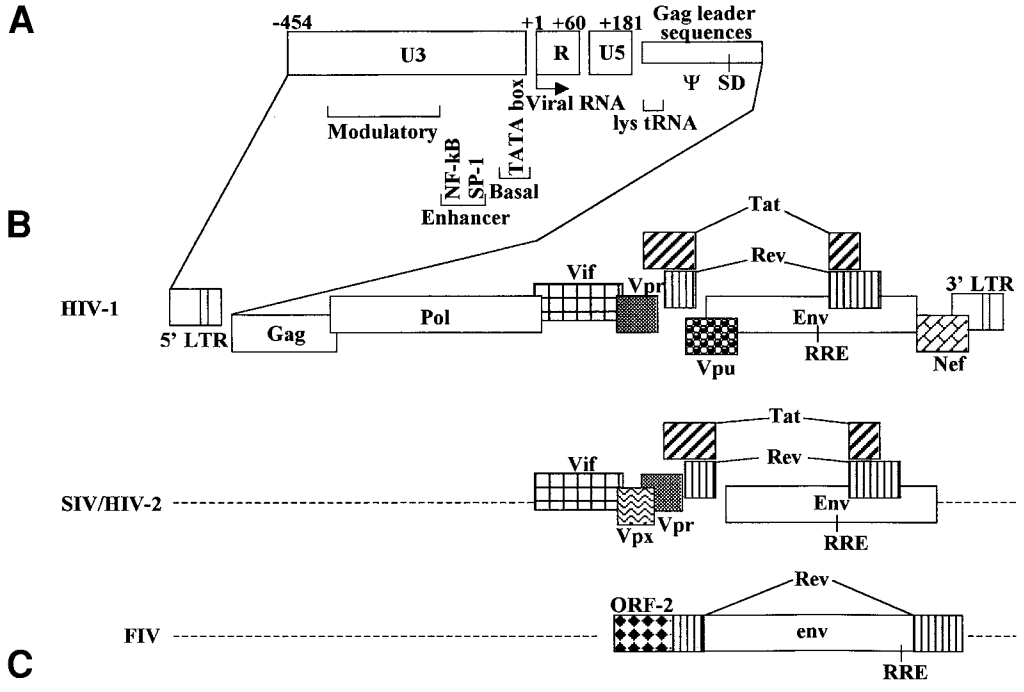
family whose genomes are most frequently utilized for the construction of lentiviral vectors, i.e., HIV-2, simian immunodeficiency virus (SIV), and feline immunodeficiency virus (FIV) (**Fig. 1**). However, it should be considered that the genomes of less popular lentiviruses (EIAV, caprine arthritis-encephalitis virus, bovine leukemia virus, and foamy virus) have been utilized in designing alternative lentivirus vectors.

2. Structure of the Viral Genome

The length of the provirus (i.e., the viral cDNA integrated in the host genome) of lentiviruses averages 9 to 10 kilobases. Similarly to other components of the *Retroviridae* family, both ends of the lentiviral proviruses are constituted by homologous regions of 600 to 900 nucleotides (long terminal repeats—LTRs) required for virus replication, integration, and expression (**Fig. 1**). Proviral LTRs are schematically divided in three regions, U3, R, and U5, in which the first nucleotide of the R region corresponds to the transcription initiation (**Fig. 1A**). This implies that the structure of viral genomic RNA does not fully overlap that of provirus. In particular, the genomic RNA retains the U5 and R LTR regions at its 5′ end, and the U3 and part of the R region (to the polyadenilation site) at the 3′ end, resulting in the viral genome in the R-U5-genes-U3-R structure. The viral transcription activity requires the interaction of cellular factors with sequences located in the U3 region, although additional regions comprised in both R and U5, as well as in the adjacent Gag leader sequences, bind host factors (for a review, *see* **ref. *3***). The U3 region comprises basal-, enhancer-, and modulatory-promoting elements. The R region includes sequences forming stable stem loops in the growing RNA molecules that are critically involved in the Tat-mediated transactivation (*see* **Subheading 2.3.**). Finally, LTRs also contain signals for RNA capping (at the 5′ end of transcripts) and polyadenylation in the R region.

While designing new lentiviral vectors, it should be taken into consideration that for the most part the functions of LTR segregate in distinct regions, so that, for instance, one can manipulate the promoting activity without interfering

Fig. 1. *(see facing page)* Genetic structure of prototypic lentiviruses, and functions of regulatory proteins. **(A)** Structure of the proviral HIV-1 5′ LTR and Gag leader sequences. The locations of the modulatory, enhancer, and basal promoter elements in the U3 region are indicated. The positions of the Lys tRNA3 binding site, the packaging (Ψ) region, and the major splice donor (SD) site in the Gag leader sequences are also reported. **(B)** Structure of the HIV-1 as compared to HIV-2/SIV and FIV genomes is shown. Major structural differences with respect to HIV-2/SIV or FIV are indicated underneath. Lengths of genes are not in scale. **(C)** Summary of the functions of the lentiviral regulatory proteins.



Viral Infectivity Factor: blocks a cellular inhibitor of viral replication

Viral Protein R: present in HIV-1 only. It participates in the migration of the preintegration complex to the nucleus. It arrests cells in G2 in both HIV-1/2 and SIV

TrAscripTion factor: required for the efficient elongation of nascent viral transcripts

REgulatory Viral protein: binds RRE sequences, allowing the cytoplasmic export of unspliced and singly spliced viral RNAs

Viral Protein U: localized at the cell membrane, it facilitates the viral release

NEgative Factor: despite its originary definition, it promotes both viral release and infectivity

Viral Protein X: involved in the migration of the preintegration complex towards the nucleus

Open Reading Frame-2: promotes the viral genome transcription, through a TAR independent mechanism

with the vector replication and integration. From a biosafety point of view, a major concern in transducing cells with lentivirus vectors is represented by the presence at the 3′ end of a full copy of the viral promoter that, once integrated in the host genome, should theoretically switch the transcription of downstream cell genes, leading to undesired gene deregulation. Hence, in lentivirus vectors of the last generation, self-inactivating (SIN) vectors, conceived in the perspective of a clinical utilization, the basal/enhancer elements of the lentiviral promoters were efficiently replaced by transcriptional control elements from heterologous viral or cellular promoters.

The genome of lentiviruses codes for three structural precursor proteins, namely Gag, Pol, and Env (**Fig. 1B**). In HIV-1, products of *gag* and *pol* genes originate from p55 Gag and p160 Gag-Pol polypeptide precursors, respectively, which are cleaved into respective mature products by the viral protease during or immediately after the virus budding. In particular, the cleavage of the 55-kDa Gag precursor generates matrix (p17 MA), capsid (p24 CA), nucleocapsid (p9 NC), and p6 proteins, plus two spacer peptides (for a review, *see* **ref. *4***). On the other hand, the processing of the 160-kDa Gag-Pol precursor generates, besides each Gag mature product, viral protease (p12 PR), deputed to the cleavage of Gag-Pol precursors, reverse transcriptase (p51/66 RT), an enzyme playing a unique role in the synthesis of the viral cDNA from the genomic RNA, and integrase (p31 IN), required for the integration of viral DNA into the host genome (for a review, *see* **ref. *5***). Both Gag and Gag-Pol precursors are generated by the full-length viral RNA, the latter being generated by a ribosomal frameshift occurring at a rather lower rate, i.e., in a 1:20 ratio with respect to the p55 Gag precursor.

The *env* gene codes for a 160-kDa precursor, whose cleavage, driven by cell proteases, gives rise to two highly glycosylated products, i.e., the viral surface (SU) and the transmembrane (TM) envelope glycoproteins. These are involved in the cell receptor recognition and in the fusion of viral to cell membranes, respectively (for a review, *see* **ref. *6***).

Notably, lentiviruses differ from retroviruses in the presence of a set of smaller open reading frames coding for a number of accessory or, more appropriately, regulatory genes, whose functions in the viral life cycle are summarized in **Fig. 1C**. From the point of view of the lentivirus gene engineering strategies, the influence of the expression of regulatory genes on the vector transduction efficiency varies greatly with respect to the cell system considered. For instance, while recent studies showed that the expression of only HIV-1 Rev in the packaging construct is sufficient to obtain lentiviral particles able to transduce growth-arrested cell lines ***(7)***, an efficient transduction of quiescent ex vivo lymphocytes has been achieved exclusively upon the expression of all

the regulatory HIV-1 proteins in the packaging cells ***(8)***. Hence, a summary of the effects of each regulatory protein on both the viral replication and the transduction driven by the lentiviral vectors should be of some utility.

2.1. Vif Protein

The *vif* gene is present in all known lentiviruses, except EIAV, and is invariably located between *pol* and *env* genes. HIV-1 Vif is a 23-kDa protein whose expression is absolutely required for the viral replication in ex vivo cells and in few cell lines ("nonpermissive cells"). Conversely, most parts of the cell lines support the replication of HIV-1 even in absence of Vif ("permissive cells") (for a review, *see* **ref. *9***). Vif acts presumably by counteracting the effect of a cellular factor inhibiting the viral replication that was recently identified ***(10)***. It was reported that the Vif expression in lentivirus vector-producing cells improves the vector infectivity in liver cells ***(11)***.

2.2. Vpr, Vpx Proteins

Vpr is a 10-kDa virionic protein present only in HIV-1/2 and SIV and is involved in the cell cycle arrest at the G_2 stage, as well as in the migration toward the nucleus of the viral preintegration complex (PIC) (for reviews, *see* **refs. *12*** and ***13***). In HIV-2 and SIV, the latter function was correlated with the presence of the evolutionary related Vpx protein (for a review, *see* **ref. *14***).

The Vpr expression in the producer cells significantly increases the vector infectivity in human monocyte-derived macrophages, possibly by facilitating the nucleus incoming of the lentivector PIC ***(15–18)***. In addition, a reduced rate in the mutation frequency of transduced sequences has been recently associated with either the Vpr presence in the vector viral particles or its expression in target cells ***(19,20)***.

2.3. Tat Protein

HIV-1 Tat is a 15-kDa protein that is strictly required for the replication of lentiviruses and whose net effect is a dramatic enhancement of the rate of viral genome transcription. It is now well established that the transcriptional transactivation mediated by Tat acts mostly at the level of elongation of viral transcripts. While interacting with a set of cellular factors like Cyclin T1, CDK9, Creb-binding protein, p300, and NF-κB, Tat binds a stem loop region (TAR, for Tat activating region) generated by the growing viral RNA and comprised in the R region of the 5′ LTR (for a review, *see* **ref. *21***). Several reports assign to Tat additional characteristics and functions. In particular, Tat could directly bind the elongating RNA polymerase II ***(22)*** and/or DNA-tethered promoter factors ***(23)***. In addition, Tat can relieve the transcription-

ally inactive proviral HIV genome by means of the recruitment of histone acetyltransferases ***(24)*** and increases the transcription of cell genes through a TAR-independent mechanism, i.e., by acting as a DNA sequence-specific transcription factor ***(25)***. Worthy of note, in the FIV genome, the transactivating function depends on ORF-2, a protein structurally similar to Tat, but acting through alternative mechanisms ***(26)***.

The expression of Tat is required when the packaging construct and/or the lentiviral vector are promoted by lentiviral LTR. However, vectors of last generations lack the Tat dependence by means of regulating the expression of the packaging constructs by heterologous promoters and by using transfer vectors that retain only the replicative functions of LTR. This should be considered as a significant advantage, as Tat interferes with several cellular functions.

2.4. Rev Protein

HIV-1 Rev is a 21-kDa protein whose shuttling properties are involved in the nucleus to cytoplasm translocation of lentiviral transcripts. The HIV-1 genome is expressed by means of the transcription of three families of RNAs: multispliced RNAs for early proteins (Nef, Tat, and Rev); single-spliced RNAs for the expression of Vif, Vpr, Vpu, and Env; and unspliced RNA for the expression of both Gag and Gag-Pol precursors (for a detailed treatment of splicing mechanisms in HIV-1, *see* **ref.** ***27***). Genomes of lentiviruses hold sequences, INS (instability sequences), mainly within the structural genes, inducing retention and degradation of both unspliced and singly spliced viral transcripts into the nucleus. Such a degradation effect is counteracted by the binding of Rev with a specific region, RRE (Rev responsive elements), comprised in the *env* gene (for reviews, *see* **refs.** ***28*** and ***29***). To be effective, Rev must interact with specific cellular factors, among which exportin-1 (CRM-1) ***(30)***, eIF-5A ***(31)***, Rip/Rab ***(32,33)***, and Sam 68 ***(34)*** are the best characterized. The lack of the interaction with a full complement of such factors, as occurs in rodent cells, leads to a very inefficient expression of the HIV genome (for a review, *see* **ref.** ***35***). The outcome is that, in the presence of Rev, both unspliced and single-spliced viral RNAs are efficiently exported in the cytoplasm. Otherwise, only multispliced viral RNAs (codifying for Rev itself, Tat, and Nef) could egress from the nucleus and be translated.

For these reasons, the stability of both lentiviral packaging constructs and of transfer vectors containing INS requires the Rev–RRE interaction. Lentivirus vectors in which the Rev-RRE requirement was bypassed by inserting mouse- or simian-derived CTE (constitutive transport elements) ***(36)*** sequences, whose presence in the natural host stabilizes the incompletely spliced lentiviral RNAs, led to unsatisfactory results ***(37)***.

2.5. Vpu Protein

The *vpu* gene was found in HIV-1 only. It expresses a 16-kDa type I integral membrane protein forming ion channels whose presence improves the efficiency of the viral particle release (for a review, *see* **ref. *38***). Data describing influences of Vpu on the overall efficiency of lentivirus vector-driven transduction have not been reported yet.

2.6. Nef Protein

The Nef expression increases the virus infectivity through mechanisms not completely clarified yet (for reviews, *see* **refs. *39*** and ***40***). Nef is a 27- to 34-kDa protein, myristoylated at its N-terminus, and is present exclusively in HIV-1/2 and SIV. The Nef requirement for optimal HIV-1 infectivity lacks its relevance in lentivirus vectors with viral receptors leading to a pH-dependent viral entry (as for the G glycoprotein from the vesicular stomatitis virus, or VSV-G), as their incoming was basically independent of the Nef presence ***(40,41)***. This characteristic represents a great benefit for target cells, in view of the several anticellular effects described for Nef.

3. Lentivirus Life Cycle

The critical intervention of the regulatory proteins during the viral replication is a hallmark distinguishing the life cycle of lentiviruses from that of the other members of the *Retroviridae* family. Lentiviral particles attach target cells (typically T and B lymphocytes, macrophages, astrocytes, and microglial cells) through the coordinated interactions of their envelope glycoproteins with specific cell receptors, most commonly CD4 for the primate lentiviruses, CD9 for FIV, and a chemokine receptor, CXCR4, CCR5 (for a review, *see* **ref. *42***). The incoming of viral capsid into a target cell occurs through the fusion of the viral envelope with the cell membrane in a pH-independent manner. Once delivered into the cytoplasm, the viral capsid disassembles, and the retrotranscription process starts, leading to the formation of a double-strand viral DNA. The reverse transcription appears as a very complex process (for a detailed description, *see* **ref. *43***) and is driven by a product of the *pol* gene, the reverse transcriptase, showing both RNA- and DNA-dependent DNA polymerase, as well as RNAse H, activities. The DNA synthesis is primed by a cellular transfer RNA for lysine (tRNALys3), which binds to complementary sequences in the Gag leader sequences (**Fig. 1A**). Once retrotranscribed, the 5′ LTR jumps to the R region at the 3′ end of the genome, starting a process ultimately leading to the formation of a RNA/DNA hybrid molecule, whose RNA component is progressively degraded by means of the RNase H activity of the RT enzyme. The lentivirus retrotranscription process implies that both

LTRs synthesized in the target cell originate from the 3′ LTR present in the producer one (i.e., the packaging cell, in the case of lentivirus vectors, *see* **Subheading 4.**). This should be taken into critical consideration when designing lentivirus vectors modified in the regulatory sequences.

The DNA/DNA viral double-strand forms, together with MA, IN, and Vpr viral proteins and cellular factors, the preintegration complex. The feature best distinguishing lentiviruses from the other retroviruses is the ability to integrate its cDNA independently of the cell duplication and thus to the nuclear membrane disassembling. In HIV-1, both MA and IN proteins carry typical nuclear localization sequences recognized by the importin α cell protein. Such an interaction allows the binding with the importin β, a cell protein able to target the PIC to pores of the nuclear membrane by means of the interaction with the cellular nucleoporins. Vpr participates to the PIC nuclear incoming both by increasing the affinity of importin α to the nuclear localization sequences and by acting as an analogue of importin β ***(44–47)***. Recently, a region within the *pol* gene (central DNA flap) has been found contributing as a *cis*-determinant to HIV-1 DNA nuclear import ***(48)***.

Once in the nucleus, the provirus arranges in a 2-LTR circular form and undergoes a stable integration by means of the IN activity upon cleavage at both proviral ends. Four to six nucleotides at each terminus of the viral cDNA are directly involved in the integration process, which allows the lentiviral genome to become a permanent genetic element of the host cell. Of note, the host DNA is equally accessible to the lentiviral provirus without preferential integration sites ***(49)***.

The expression of viral genes is tightly coordinated, being Nef, Tat, and Rev, the proteins codified earlier by multispliced, Rev-RRE-independent viral RNAs. Whereas the need of abundant and early production of both Tat and Rev appears pretty clear from the dynamic of lentivirus replication, the immediate appearance of large amounts of Nef still remains an intriguing matter. The Rev–RRE interaction allows the export in the cytoplasm of both unspliced and single-spliced viral RNAs, leading to the synthesis of the additional regulatory, as well as structural, viral proteins.

Late steps of the life cycle do not significantly distinguish lentiviruses from other retroviruses. Viral structural proteins reaching the cell membrane, together with the viral genome and other accessory molecules (e.g., lysine tRNA3, Vpr, and Nef proteins), assemble into mature viral particles after, or in concomitance with, the cleavage of Gag, Gag-Pol, and Env precursors in their final products. Even if, as is generally believed, most of the HIV-1 Gag processing occurs at the cell membrane, the cleavage of Gag precursors have also been described in the cell cytoplasm ***(50)***. The outcome is a mature

lentivirus particle containing two identical copies of full-length viral RNA bound by complementary sequences in the U5 region of the 5′ LTR.

4. Recovery of Recombinant Lentivirus Particles

A rather detailed knowledge of the biology of the lentivirus proved to be of critical importance for the development of the lentivirus vector technology. The theoretical approach in designing lentivirus vectors was not conceptually new, resembling in many aspects the application successfully undertaken for the development of oncoretrovirus-based vectors. However, the presence of regulatory proteins rendered the design of lentivirus vectors more complex.

The use of lentivirus-based transfer techniques relies on the in vitro production of recombinant lentiviral particles carrying a highly deleted viral genome in which the transgene of interest should be accommodated. In particular, the recombinant lentivirus particles are recovered through the *in trans* coexpression in a permissive cell line (packaging cells) of (1) the packaging construct, i.e., a vector expressing the Gag-Pol precursors together with Rev (alternatively expressed *in trans*); (2) a vector expressing an Env receptor, generally of an heterologous nature; and (3) the transfer vector, consisting in the viral cDNA deprived of all open reading frames, but maintaining the sequences required for replication, incapsidation, and expression, in which the sequences to be engineered are inserted. Of note, the transcripts for the structural proteins being devoid of the sequences involved in the packaging process (i.e., Ψ site), lentiviral particles incorporate exclusively the RNA from the transfer vector.

Although recombinant lentiviral vectors are commonly recovered by means of transient triple transfections of highly transfectable cell lines (e.g., human embryonic kidney 293 cells, or derivative thereof), considerable efforts have been made attempting to isolate an efficient packaging cell clone from which lentivirus particles should be obtained through the transfection of the transfer vector only ***(51,52)***. In any case, the vector infects target cells through a single cycle, abortive infection, and stably integrates into the host genome without need of cell replication. This process opens the way toward new applications in an array of even postmitotic cell types, as described in much detail in the chapters of this book.

5. Conclusion

Because the highly pathogenic nature of replication competent lentiviruses, last generations of lentivirus vectors have been conceived in a continuous effort to minimize the presence of unnecessary viral sequences, both in the packaging and in the vector constructs. Likely, the next fascinating frontier

is represented by the accomplishment of the in vivo delivery of therapeutic transgenes through the use of both targetable and injectable lentivirus vectors, as anticipated by some encouraging but still preliminary results ***(53–55)***.

The aim of the present book is to go through the experimental details of the most significant applications successfully carried out by using various generation of lentivirus vectors. It is both surprising and exciting to learn how different and effective applications have been accomplished by means of the lentivirus vector-mediated engineering.

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References

1. Vallee, H. and Carré, H. (1904) Nature infectieuse de l'anemie de cheval. *C. R. Acad. Sci.* **139,** 331–333.
2. Coffin, J. M., Huges, S. H., and Varmus, H. E. (1997) Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
3. Pereira, L. A., Bentley, K., Peeters, A., Churchill, M. J., and Deacon, N. J. (2000) A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. *Nucleic Acids Res.* **28,** 663–668.
4. Freed, E. O. (1998) HIV-1 gag proteins: diverse functions in the virus life cycle. *Virology* **251,** 1–15.
5. Li, X., Quan, Y., and Wainberg, M. A. (1997) Controlling elements in replication of the human immunodeficiency virus type 1. *Cell Mol. Biol. (Noisy. -le-grand)* **43,** 443–454.
6. Wyatt, R. and Sodroski, J. (1998) The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* **280,** 1884–1888.
7. Kim, V. N., Mitrophanous, K., Kingsman, S. M., and Kingsman, A. J. (1998) Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. *J. Virol.* **72,** 811–816.
8. Chinnasamy, D., Chinnasamy, N., Enriquez, M. J., Otsu, M., Morgan, R. A., and Candotti, F. (2000) Lentiviral-mediated gene transfer into human lymphocytes: role of HIV-1 accessory proteins. *Blood* **96,** 1309–1316.
9. Inubushi, R. and Adachi, A. (1999) Cell-dependent function of HIV-1 Vif for virus replication (Review). *Int. J. Mol. Med.* **3,** 473–476.
10. Sheehy, A. M., Gaddis, N. C., Choi, J. O., and Malim, M. H. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral VIF protein. *Nature* **418,** 646–650.

11. Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H., and Verma, I. M. (1997) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat. Genet.* **17,** 314–317.
12. Bukrinsky, M. and Adzhubei, A. (1999) Viral protein R of HIV-1. *Rev. Med. Virol.* **9,** 39–49.
13. Elder, R. T., Benko, Z., and Zhao, Y. (2002) HIV-1 VPR modulates cell cycle G2/M transition through an alternative cellular mechanism other than the classic mitotic checkpoints. *Front. Biosci.* **7,** d349–d357.
14. Kappes, J. C. (1995) Viral protein X. *Curr. Top. Microbiol. Immunol.* **193,** 121–132.
15. Connor, R. I., Chen, B. K., Choe, S., and Landau, N. R. (1995) Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. *Virology* **206,** 935–944.
16. Mahalingam, S., Ayyavoo, V., Patel, M., Kieber-Emmons, T., and Weiner, D. B. (1997) Nuclear import, virion incorporation, and cell cycle arrest/differentiation are mediated by distinct functional domains of human immunodeficiency virus type 1 Vpr. *J. Virol.* **71,** 6339–6347.
17. Eckstein, D. A., Sherman, M. P., Penn, M. L., et al. (2001) HIV-1 Vpr enhances viral burden by facilitating infection of tissue macrophages but not nondividing CD4+ T cells. *J. Exp. Med.* **194,** 1407–1419.
18. De Noronha, C. M., Sherman, M. P., Lin, H. W., et al. (2001) Dynamic disruptions in nuclear envelope architecture and integrity induced by HIV-1 Vpr. *Science* **294,** 1105–1108.
19. Jowett, J. B., Xie, Y. M., and Chen, I. S. (1999) The presence of human immunodeficiency virus type 1 Vpr correlates with a decrease in the frequency of mutations in a plasmid shuttle vector. *J. Virol.* **73,** 7132–7137.
20. Mansky, L. M. (1996) The mutation rate of human immunodeficiency virus type 1 is influenced by the vpr gene. *Virology* **222,** 391–400.
21. Marcello, A., Zoppe, M., and Giacca, M. (2001) Multiple modes of transcriptional regulation by the HIV-1 Tat transactivator. *IUBMB Life* **51,** 175–181.
22. Jones, K. A. (1997) Taking a new TAK on tat transactivation. *Genes Dev.* **11,** 2593–2599.
23. Cujec, T. P., Cho, H., Maldonado, E., Meyer, J., Reinberg, D., and Peterlin, B. M. (1997) The human immunodeficiency virus transactivator Tat interacts with the RNA polymerase II holoenzyme. *Mol. Cell Biol.* **17,** 1817–1823.
24. Benkirane, M., Chun, R. F., Xiao, H., et al. (1998) Activation of integrated provirus requires histone acetyltransferase. p300 and P/CAF are coactivators for HIV-1 Tat. *J. Biol. Chem.* **273,** 24,898–24,905.
25. Roebuck, K. A., Rabbi, M. F., and Kagnoff, M. F. (1997) HIV-1 Tat protein can transactivate a heterologous TATAA element independent of viral promoter sequences and the transactivation response element. *AIDS* **11,** 139–146.
26. de Parseval, A. and Elder, J. H. (1999) Demonstration that orf2 encodes the feline immunodeficiency virus transactivating (Tat) protein and characterization of a unique gene product with partial rev activity. *J. Virol.* **73,** 608–617.

27. Purcell, D. F. and Martin, M. A. (1993) Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. *J. Virol.* **67,** 6365–6378.
28. Pollard, V. W. and Malim, M. H. (1998) The HIV-1 Rev protein. *Annu. Rev. Microbiol.* **52,** 491–532.
29. Fukumori, T., Kagawa, S., Iida, S., et al. (1999) Rev-dependent expression of three species of HIV-1 mRNAs (review). *Int. J. Mol. Med.* **3,** 297–302.
30. Yi, R., Bogerd, H. P., and Cullen, B. R. (2002) Recruitment of the Crm1 nuclear export factor is sufficient to induce cytoplasmic expression of incompletely spliced human immunodeficiency virus mRNAs. *J. Virol.* **76,** 2036–2042.
31. Ruhl, M., Himmelspach, M., Bahr, G. M., et al. (1993) Eukaryotic initiation factor 5A is a cellular target of the human immunodeficiency virus type 1 Rev activation domain mediating trans-activation. *J. Cell Biol.* **123,** 1309–1320.
32. Bogerd, H. P., Fridell, R. A., Madore, S., and Cullen, B. R. (1995) Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. *Cell* **82,** 485–494.
33. Fritz, C. C., Zapp, M. L., and Green, M. R. (1995) A human nucleoporin-like protein that specifically interacts with HIV Rev. *Nature* **376,** 530–533.
34. Reddy, T. R., Xu, W., Mau, J. K., et al. (1999) Inhibition of HIV replication by dominant negative mutants of Sam68, a functional homolog of HIV-1 Rev. *Nat. Med.* **5,** 635–642.
35. Boris-Lawrie, K., Roberts, T. M., and Hull, S. (2001) Retroviral RNA elements integrate components of post-transcriptional gene expression. *Life Sci.* **69,** 2697–2709.
36. Zolotukhin, A. S., Valentin, A., Pavlakis, G. N., and Felber, B. K. (1994) Continuous propagation of RRE(-) and Rev(-)RRE(-) human immunodeficiency virus type 1 molecular clones containing a *cis*-acting element of simian retrovirus type 1 in human peripheral blood lymphocytes. *J. Virol.* **68,** 7944–7952.
37. Gasmi, M., Glynn, J., Jin, M. J., Jolly, D. J., Yee, J. K., and Chen, S. T. (1999) Requirements for efficient production and transduction of human immunodeficiency virus type 1-based vectors. *J. Virol.* **73,** 1828–1834.
38. Bour, S. and Strebel, K. (2000) HIV accessory proteins: multifunctional components of a complex system. *Adv. Pharmacol.* **48,** 75–120.
39. Geyer, M., Fackler, O. T., and Peterlin, B. M. (2001) Structure—function relationships in HIV-1 Nef. *EMBO Rep.* **2,** 580–585.
40. Arold, S. T. and Baur, A. S. (2001) Dynamic Nef and Nef dynamics: how structure could explain the complex activities of this small HIV protein. *Trends Biochem. Sci.* **26,** 356–363.
41. Chazal, N., Singer, G., Aiken, C., Hammarskjold, M. L., and Rekosh, D. (2001) Human immunodeficiency virus type 1 particles pseudotyped with envelope proteins that fuse at low pH no longer require Nef for optimal infectivity. *J. Virol.* **75,** 4014–4018.
42. Clapham, P. R. and McKnight, A. (2001) HIV-1 receptors and cell tropism. *Br. Med. Bull.* **58,** 43–59.

43. Jonckheere, H., Anne, J., and De Clercq, E. (2000) The HIV-1 reverse transcription (RT) process as target for RT inhibitors. *Med. Res. Rev.* **20,** 129–154.
44. Gallay, P., Hope, T., Chin, D., and Trono, D. (1997) HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc. Natl. Acad. Sci. USA* **94,** 9825–9830.
45. Popov, S., Rexach, M., Ratner, L., Blobel, G., and Bukrinsky, M. (1998) Viral protein R regulates docking of the HIV-1 preintegration complex to the nuclear pore complex. *J. Biol. Chem.* **273,** 13,347–13,352.
46. Jenkins, Y., McEntee, M., Weis, K., and Greene, W. C. (1998) Characterization of HIV-1 vpr nuclear import: analysis of signals and pathways. *J. Cell Biol.* **143,** 875–885.
47. Haffar, O. K., Popov, S., Dubrovsky, L., et al. (2000) Two nuclear localization signals in the HIV-1 matrix protein regulate nuclear import of the HIV-1 pre-integration complex. *J. Mol. Biol.* **299,** 359–368.
48. Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., and Charneau, P. (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101,** 173–185.
49. Katzman, M. and Katz, R. A. (1999) Substrate recognition by retroviral integrases. *Adv. Virus Res.* **52,** 371–395.
50. Kaplan, A. H. and Swanstrom, R. (1991) Human immunodeficiency virus type 1 gag proteins are processed in two cellular compartments. *Proc. Natl. Acad. Sci. USA* **88,** 4528–4532.
51. Farson, D., Witt, R., McGuinness, R., et al. (2001) A new-generation stable inducible packaging cell line for lentiviral vectors. *Hum. Gene Ther.* **12,** 981–997.
52. Xu, K., Ma, H., McCown, T. J., Verma, I. M., and Kafri, T. (2001) Generation of a stable cell line producing high-titer self-inactivating lentiviral vectors. *Mol. Ther.* **3,** 97–104.
53. Seppen, J., Barry, S. C., Harder, B., and Osborne, W. R. (2001) Lentivirus administration to rat muscle provides efficient sustained expression of erythropoietin. *Blood* **98,** 594–596.
54. Baek, S. C., Lin, Q., Robbins, P. B., Fan, H., and Khavari, P. A. (2001) Sustainable systemic delivery via a single injection of lentivirus into human skin tissue. *Hum. Gene Ther.* **12,** 1551–1558.
55. Peng, K. W., Pham, L., Ye, H., et al. (2001) Organ distribution of gene expression after intravenous infusion of targeted and untargeted lentiviral vectors. *Gene Ther.* **8,** 1456–1463.

2

The Choice of a Suitable Lentivirus Vector

Transcriptional Targeting

Francesco Lotti and Fulvio Mavilio

1. Oncoretroviral and Lentiviral Vectors

Human immunodeficiency virus (HIV)-derived lentiviral vectors can integrate into the genome of dividing and nondividing cells, in vitro as well as in vivo (reviewed in **refs. *1–3***). Lentiviral vectors are particularly efficient in transducing multipotent stem cells, such as hematopoietic stem cells (HSC), without compromising their self-renewing and organ repopulation capacity upon transplantation in vivo ***(4–6)***. This is a crucial advantage over oncoretroviral vectors derived from the Moloney murine leukemia virus (MoMLV) since transplantation of genetically modified stem cells (hematopoietic, neural, or epidermal) is a potential therapy for a variety of genetic and acquired disorders, including diabetes, multiple sclerosis, cancer, and acquired immunodeficiency syndrome (AIDS). Several years of research have drastically improved both design and packaging of lentiviral vectors, minimized the use of HIV structural and regulatory sequences in both the transfer and the packaging constructs and have virtually abolished the safety concerns originally raised by the idea of transducing human cells with a derivative of HIV (reviewed in **ref. *3***). These vectors are now a very promising alternative to transduce transplantable stem cells ex vivo and such postmitotic tissues as the central nervous system (CNS) or liver in vivo.

2. Targeting of Retroviral Vectors

For most clinical applications, restricting the expression of a retrovirally delivered therapeutic transgene to a specific subset of cells within a tissue, or

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to a specific progeny of a multipotent stem cell, is a mandatory requirement. This need can be achieved either by targeting the vector entry into a specific cell subset (transductional targeting) and/or by targeting the expression of the transgene by appropriately regulating its transcription (transcriptional targeting). Redirecting retroviral particle entry can be achieved by either pseudotyping, genetically modifying the envelope glycoprotein, or by inserting soluble adaptors between the viral particle and the desired target cell. Proof of principle for this type of technology has been provided in the past for both oncoretroviral and lentiviral vectors. However, increasing specificity, while maintaining infectivity of a retroviral particle, turned out to be difficult, and the entire technology is still far from meeting the safety and efficacy requirements for real clinical application (reviewed in **ref. *7***). On the other hand, redirecting the transcriptional properties of retroviral vectors has proven a relatively easier task, although the RNA nature and the limited size of a retroviral genome impose significant constrains on the complexity of the regulation that can be achieved in both oncoretroviral and lentiviral vectors.

Restriction of transgene expression in the context of an oncoretroviral vector has been obtained by using tissue-specific or exogenously regulated promoters and regulatory elements (reviewed in **refs. *7*** and ***8***). Transcription of an integrated MoMLV provirus is normally dependent upon the viral promoter/enhancer elements located in the U3 region of the 5′ long terminal repeat (LTR), which allows constitutive expression of a transferred gene in most cell types. An independently controlled transcriptional unit can be transferred and expressed in the framework of a MoMLV-derived vector. However, the strong activity of the LTR usually interferes with, or prevents, regulation and function of most eukaryotic cis-acting elements, even in the proper cell context ***(9,10)***. Alternative designs have been developed in an attempt to overcome this problem, from self-inactivating (SIN) vectors, in which deletion of viral elements allows expression from an internal promoter ***(11–13)***, to vectors in which a transgene is transcribed in opposite orientation with respect to the viral transcription unit ***(14)***, and also to "double-copy" vectors in which a complete minigene is inserted into the LTR upstream of the U3 region ***(15)***. Although effective, these strategies often raise other problems, such as generation of antisense transcripts, proviral instability, or decreased viral titers. Alternatively, the promiscuous MoMLV LTR transcriptional activity can be redirected by adding to or replacing the LTR U3 region with cellular enhancers, effectively restricting transgene expression to specific tissues or cell lineages ***(16,17)***.

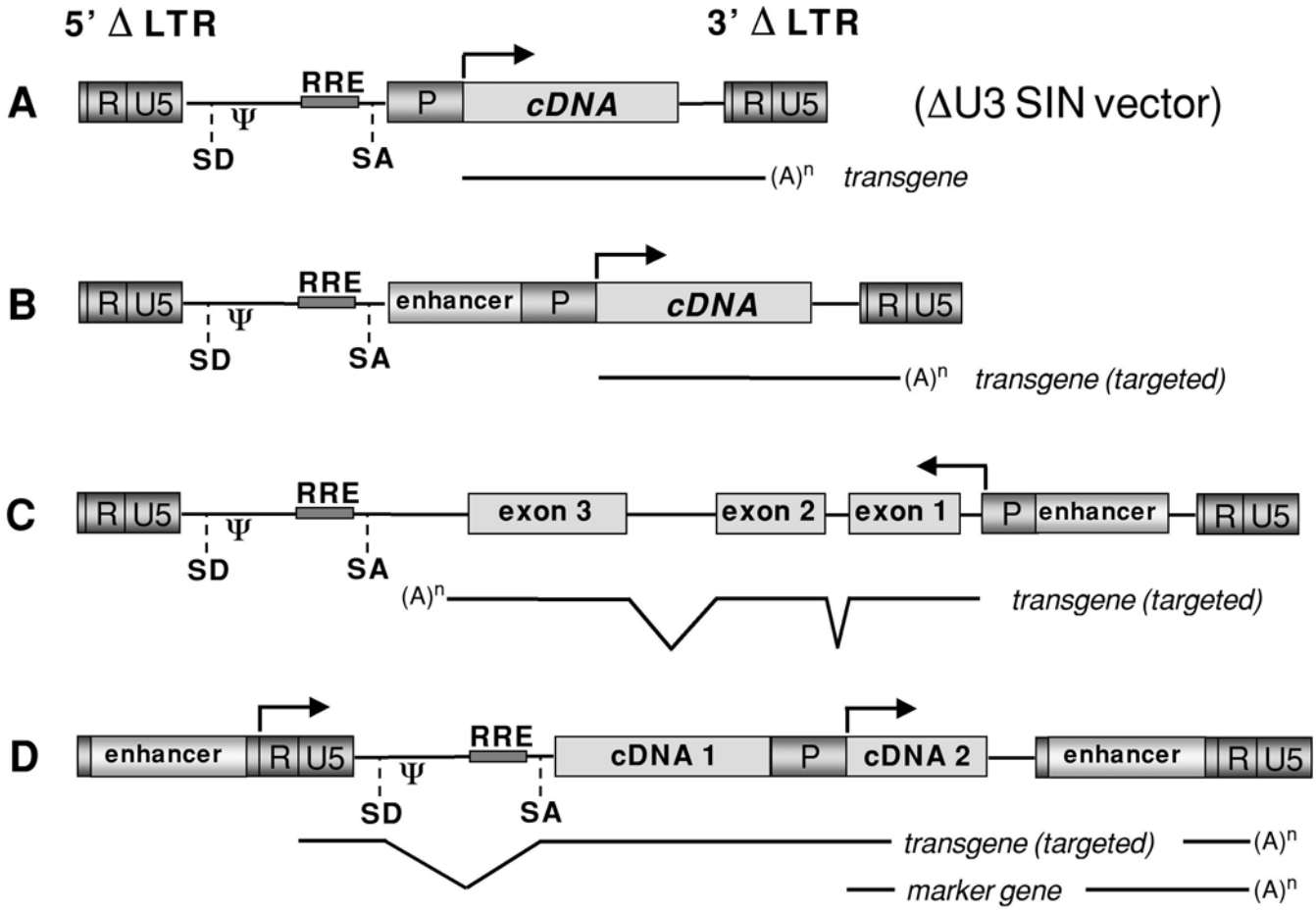
3. Transcriptional Targeting of Lentiviral Vectors

Transcriptional targeting of lentiviral vectors meets essentially the same type of technical difficulties encountered with oncoretroviral vectors. However,

transcriptional interference is usually not a problem in a lentiviral context since the SIN design, i.e., the complete elimination of the 5′ LTR enhancer and basal promoter (**Fig. 1A**), is mandatory to minimize the regions of potential overlapping, and therefore recombination, between the transfer vector and the packaging constructs ***(18,19)***. This allows the use of internal promoters combined to tissue- or cell-specific enhancers to regulate transgene expression. With this simple design (**Fig. 1B**), expression of a reporter or a therapeutic transgene has been effectively restricted to erythroblasts ***(20,21)***, or antigen-presenting cells ***(22)*** derived from murine or human HSCs by the use of erythroid-specific enhancers driving the ankyrin-1 promoter or the human HLA-DRα promoter, respectively. Attempts have also been made to introduce exogenously regulated transcriptional elements into lentiviral vectors, such as a tetracycline-responsive element linked to a minimal promoter to drive the gene of interest, and different variants of tetracycline-regulated, chimeric transcriptional activators ***(23,24)***. With these vectors, the activation of a reporter or a therapeutic gene has been obtained both in vitro and in vivo, in particular in rats injected in the brain with the vector or in mice transplanted with transduced HSCs, by simply adding or withdrawing the tetracycline analog doxicycline from the animals' drinking water. Although these studies are encouraging and prove the principle that exogenously regulated transcription is achievable in the context of a lentiviral vector, the use of Tet-responsive or analogous hormone-inducible systems in gene therapy applications will require the development of "humanized," nonimmunogenic transcriptional activators, possibly controlled by innocuous drugs.

The major limitation of using internal transcription units within lentiviral vectors is the absence of introns in the sub genomic transcript used to express the gene of interest, which significantly affects its posttranscriptional fate (polyadenylation, nuclear export, stability) and ultimately reduces its efficacy in terms of protein output. For some gene therapy applications, e.g., the correction of globin gene imbalance in human β-thalassemia, a high protein output is just as important as its restricted expression, which makes practically inadequate the use of intronless β-globin cDNAs expressed by internal promoters. This problem has been partially resolved by placing the entire human β-globin gene under the control of its own promoter and a reduced version of the β-globin locus control region (LCR) in opposite orientation within a SIN vector. With this design (**Fig. 1C**), potentially therapeutic levels of human β-globins have been obtained for the first time in a murine model of β-thalassemia ***(25,26)***.

As in the case of oncoretroviral vectors, transcriptional targeting of lentiviral vectors can be conveniently achieved by LTR enhancer replacement. An erythroid-specific enhancer (the GATA-1 HS2 upstream element) was recently


5' Δ LTR
3' Δ LTR
A
R U5
RRE
Ψ
SD
SA
P
cDNA
R U5
(ΔU3 SIN vector)
(A)n
transgene
B
R U5
RRE
Ψ
SD
SA
enhancer
P
cDNA
R U5
(A)n
transgene (targeted)
C
R U5
RRE
Ψ
SD
SA
exon 3
exon 2
exon 1
P
enhancer
R U5
(A)n
transgene (targeted)
D
enhancer
R U5
RRE
Ψ
SD
SA
cDNA 1
P
cDNA 2
enhancer
R U5
transgene (targeted)
(A)n
marker gene
(A)n

used to replace most of the HIV LTR U3 region immediately upstream of the viral promoter. The modified LTR was used to drive the expression of a reporter gene, while a second gene was placed under the control of an internal constitutive promoter to monitor cell transduction, or immunoselect transduced cells, independent of the expression of the targeted promoter **(Fig. 1D)**. The transcriptionally targeted vector directed high levels of transgene expression specifically in mature erythroblasts derived from human and murine HSCs in vivo in a Tat-independent fashion and with no alteration in titer, infectivity, and genomic stability of the vector. Expression from the modified LTR was higher and better restricted than that obtained by the same combination of enhancer/promoter elements placed in a conventional, internal position *(27)*. A critical advantage of this targeting strategy is the use of the spliced major viral transcript to express the gene of interest, with the procedure partially overcoming the major limitation of the internal promoter design. The internal, less efficient, transcription unit can nevertheless be used to independently express a second gene providing an additional function, e.g., an in vivo selectable marker, to the transduced cells. The use of posttranscriptional regulatory elements, such as that derived from the posttranscriptional regulatory element (WPRE) the woodchuck hepatitis virus *(28)*, at alternative positions within the vector allowed the reduction of the consequences of transcriptional interference between the two enhancers and therefore modulated the expression of two transcriptional units within a single vector *(27)*.

A possible limitation of this vector design is the persistence of a potentially active, Tat-responsive HIV promoter in the integrated provirus. This element could theoretically be activated by super infection of wild-type HIV, for instance in the T-cell or macrophage progeny of transduced HSCs in an HIV-infected individual, thereby leading to mobilization of the vector genome into infective HIV particles. Although the occurrence of such an event should

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Fig. 1. Alternative lentiviral vector designs for transcriptional targeting. **(A)** Schematic representation of a SIN vector provirus in which a ubiquitous viral promoter controls the transgene expression cassette. The U3 region, containing the viral enhancer ad promoter, is deleted in both LTRs. **(B)** Transcriptional targeting of a SIN vector by an internal expression cassette that is driven by a basal promoter and a tissue- or cell-lineage-specific enhancer. **(C)** Transcriptional targeting by insertion in opposite transcriptional orientation into a SIN vector of a gene that is driven by a basal promoter and a tissue- or cell-lineage-specific enhancer and that contains one or more introns, a polyadenylation site, and 5′ and 3′ untranslated regions. **(D)** Transcriptional targeting by LTR enhancer replacement. In this design, the LTR U3 region is replaced by a tissue- or cell-lineage-specific enhancer driving the LTR viral promoter, while a second expression cassette is inserted in an internal position. The targeted transcript is spliced at the HIV Gag splice donor (SD) and acceptor (SA) sites.

be practically verified in an appropriate in vivo model, vector mobilization would be an undesirable effect, potentially affecting the safety characteristics of the gene transfer system for clinical applications. However, last-generation lentiviral packaging systems do not rely any more on Tat for the expression of the transfer vector into the packaging cell line *(29)*, allowing the design of lentiviral vectors that do not incorporate a TAR element in the primary transcript. These modifications should abolish the possibility of HIV-induced mobilization and therefore restore to enhancer-replaced vectors a degree of safety comparable to that of the fully U3-deleted SIN vectors.

An additional advantage of enhancer replacement as a targeting strategy is that the integrated provirus carries two copies of a genomic enhancer within the two LTRs **(Fig. 1D)**. The presence of two active enhancers flanking the transgene transcriptional unit could reduce the chances of chromatin-mediated inactivation of transcription, a process known to affect the long-term maintenance of retroviral transgene expression in vivo, particularly in stem cells *(30)*. Indeed, a vector containing an erythroid-specific enhancer in both LTRs showed remarkably less position effect variegation in individual cell clones than a vector based on the same enhancer/promoter combination placed in an internal position *(27)*. This vector design could therefore be indicated in all cases in which long-term persistence of gene expression at homogeneous levels independent of the integration site is required in a specific progeny of transduced HSCs. Double copies of at least some cell-specific enhancers could indeed act as transcriptional insulators, thus replacing LCRs, matrix-attachment, or other elements influencing chromatin configuration in order to shield randomly integrated transgenes from chromatin-mediated silencing. Indeed, LCR sequences do not appear to confer position-independent expression to the viral-encoded transgene *(25)*, and their role in maintaining open chromatin conformation to transcribed loci has been challenged by several authors *(31,32)*. Matrix- or scaffold-attachment elements have also been introduced in retroviral vectors to reduce positional variegation effects with only partial success *(33–36)*. Binding of tissue- or lineage-specific transcription factors to specific regulatory regions (e.g., those defined by DNase hypersensitive sites) could instead be a key factor in initiating and maintaining active chromatin structures around genes regulated in development or differentiation *(37,38)*, providing a rationale for using multiple copies of these elements in gene transfer vectors to reproduce a differentiation-specific regulation at many different genomic regions.

4. Enhancer Replacement: Vector Design and Testing

As in all retroviruses, the HIV LTR contains U3, R, and U5 elements *(39)*. The U3 region contains the viral enhancer, and it is the region that is replaced

with a tissue- or lineage-specific enhancer in this type of targeting strategy. The U3 region is conveniently deleted from positions –418/-40 to the transcription start site (+1) and is replaced by any enhancer of comparable size. This deletion leaves in place the LTR minimal promoter, which is virtually inactive in the absence of an enhancer ***(27)***. The maximum length of the sequence that can be accommodated into an U3-deleted HIV LTR is not known. By analogy with oncoretroviral vectors, it is advisable not to exceed twice the length of the removed U3 fragment (i.e., approx 800 bp) to avoid problems of genomic instability of the provirus ***(40,41)***. The nature of the inserted sequence is also important. For instance, it should not contain repeated sequences or sequences giving rise to stable secondary structures when in single-strand RNA form, although the only way to know whether a certain sequence is compatible with a stable retrovirus is to test it. The U3 region is replaced in the 3′ LTR of a second- or third-generation lentiviral vector containing a constitutive enhancer in the 5′ LTR for efficient transcription in the packaging cells ***(19)***. The LTR modification is propagated to the 5′ LTR of the integrated provirus during the reverse transcription phase into the target cells ***(39)***. A second expression cassette under the control of an independent promoter can be placed into the vector immediately downstream from the splice acceptor site **(Fig. 1D)**. The targeted vector should contain a central polypurine tract (cPPT) to enhance transduction ***(5)*** and a WPRE element upstream of the internal promoter to increase the expression of the targeted transgene or downstream of the internal expression cassette to increase the expression of both genes ***(27)***.

Enhancer-replaced vectors are packaged as regular lentiviral vectors by transient transfection into the human immortalized kidney cell line 293T of the vector plasmid, together with one or two plasmids expressing the helper *gag*, *pol*, *tat*, and *rev* genes and a plasmid expressing a suitable envelope protein, typically the G glycoprotein derived from the vesicular stomatitis virus (VSV-G).

The efficacy of the targeting is tested by transducing cells in which the targeted LTR is expected to be active and by transducing control cells in which it is expected to be silent. Transduction of both types of cells is controlled by the expression of the internal marker gene, typically GFP, *lacZ*, or a surface receptor such as ΔLNGFR ***(27)***. When the targeting is done with the purpose of expressing a transgene in a specific progeny of HSCs, the efficacy of the enhancer in restricting transgene expression to the desired progeny is tested by assaying differential gene expression in both progenitors and progeny upon differentiation in vitro and/or in vivo. Marker genes like GFP or ΔLNGFR are typically assayed by cytofluorimetry together with a lineage-specific surface marker **(Fig. 2)**, while any other gene product requires an assay with a specific antibody or, when this is unavailable, RNA analysis (Northern blotting, RNase protection, RT-polymerase chain reaction [PCR]) with specific probes or primers.

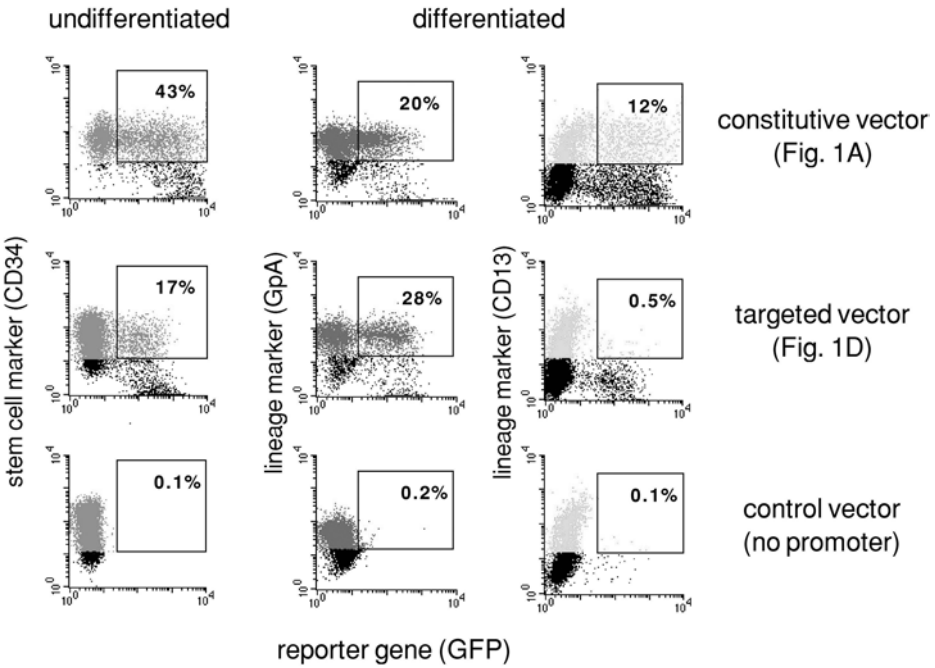

Fig. 2. Expression of a GFP (green fluorescent protein) transgene from a constitutive SIN vector (upper panels), a lentiviral vector transcriptionally targeted as in **Fig. 1D** (middle panels), and a control, promoterless vector (lower panels). In this example, the constitutive enhancer is from the cytomegalovirus (CMV), while the enhancer inserted in the LTR is a short regulatory sequence (HS2) of the erythroid-specific GATA-1 gene. Expression of GFP is assayed by fluorescence-activated cell sorter analysis of human CD34+ hematopoietic stem/progenitor cells after 3 d of culture in maintenance medium (left panels) or 10 days of culture in medium inducing either erythroid (middle panels) or myelomonocytic (right panels) differentiation. Cells were analyzed after staining with a PE (phycoerthrin)-conjugated antibody against specific surface markers for undifferentiated progenitors (CD34, right panels), erythroid cells (Glycophorin A, GpA, middle panels), or myeloid cells (CD13, right panels). Values in the framed areas indicate the percentage of cells double positive for GFP and each lineage marker. The CMV promoter is expressed in all cell types (43% of the $CD34^+$ cells, 20% of the GpA^+ cells, and 12% of the $CD13^+$ cells), while the GATA-1 targeted LTR is expressed in the CD34+ (17%) and GpA+ (28%) cells but not in CD13+ (0.5%) cells.

References

1. Naldini, L. and Verma, I. M. (1999) Lentiviral vectors, in *The Development of Gene Therapy* (Friedmann, T., ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 47–60.

2. Trono, D. (2000) Lentiviral vectors: turning a deadly foe into a therapeutic agent. *Gene Ther.* **7,** 20–23.
3. Vigna, E. and Naldini, L. (2000) Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. *J. Gene Med.* **2,** 308–316.
4. Miyoshi, H., Smith, K. A., Mosier, D. E., Verma, I. M., and Torbett, B. E. (1999) Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* **283,** 682–686.
5. Follenzi, A., Ailles, L. E., Bakovic, S., Geuna, M., and Naldini, L. (2000) Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat. Genet.* **25,** 217–222.
6. Woods, N. B., Fahlman, C., Mikkola, H., et al. (2000) Lentiviral gene transfer into primary and secondary NOD/SCID repopulating cells. *Blood* **96,** 3725–3733.
7. Larochelle, A., Peng, K. W., and Russell, S. J. (2002) Lentiviral vector targeting. *Curr. Top Microbiol. Immunol.* **261,** 143–163.
8. Miller, N. and Whelan, J. (1997) Progress in transcriptionally targeted and regulatable vectors for genetic therapy. *Hum. Gene Ther.* **8,** 803–815.
9. Emerman, M. and Temin, H. M. (1984) Genes with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism. *Cell* **39,** 449–467.
10. Emerman, M. and Temin, H. M. (1986) Quantitative analysis of gene suppression in integrated retrovirus vectors. *Mol. Cell. Biol.* **6,** 792–800.
11. Yu, S., von Ruden, T., Kantoff, P. W., et al. (1986) Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proc. Natl. Acad. Sci. USA* **83,** 3194–3198.
12. Yee, J. K., Moores, J. C., Jolly, D. J., Wolff, J. A., Respess, J. G., and Friedmann, T. (1987) Gene expression from transcriptionally disabled retroviral vectors. *Proc. Natl. Acad. Sci. USA* **84,** 5197–5201.
13. Naffakh, N., Pinset, C., Montarras, D., et al. (1996) Long-term secretion of therapeutic proteins from genetically modified skeletal muscles. *Hum. Gene Ther.* **7,** 11–21.
14. Karlsson, S., Papayannopoulou, T., Schweiger, S. G., Stamatoyannopoulos, G., and Nienhuis, A. W. (1987) Retroviral-mediated transfer of genomic globin genes leads to regulated production of RNA and protein. *Proc. Natl. Acad. Sci. USA* **84,** 2411–2415.
15. Hantzopoulos, P. A., Sullenger, B. A., Ungers, G., and Gilboa, E. (1989) Improved gene expression upon transfer of the adenosine deaminase minigene outside the transcriptional unit of a retroviral vector. *Proc. Natl. Acad. Sci. USA* **86,** 3519–3523.
16. Ferrari, G., Salvatori, G., Rossi, C., Cossu, G., and Mavilio, F. (1995) A retroviral vector containing a muscle-specific enhancer drives gene expression only in differentiated muscle fibers. *Hum. Gene Ther.* **6,** 733–742.
17. Grande, A., Piovani, B., Aiuti, A., Ottolenghi, S., Mavilio, F., and Ferrari, G. (1999) Transcriptional targeting of retroviral vectors to the erythroblastic progeny of transduced hematopoietic stem cells. *Blood* **93,** 3276–3285.

18. Zufferey, R., Dull, T., Mandel, R. J., et al. (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* **72,** 9873–9880.
19. Dull, T., Zufferey, R., Kelly, M., et al. (1998) A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* **72,** 8463–8471.
20. Richard, E., Mendez, M., Mazurier, F., et al. (2001) Gene therapy of a mouse model of protoporphyria with a self-inactivating erythroid-specific lentiviral vector without preselection. *Mol. Ther.* **4,** 331–338.
21. Moreau-Gaudry, F., Xia, P., Jiang, G., et al. (2001) High-level erythroid-specific gene expression in primary human and murine hematopoietic cells with self-inactivating lentiviral vectors. *Blood* **98,** 2664–2672.
22. Cui, Y., Golob, J., Kelleher, E., Ye, Z., Pardoll, D., and Cheng, L. (2002) Targeting transgene expression to antigen-presenting cells derived from lentivirus-transduced engrafting human hematopoietic stem/progenitor cells. *Blood* **99,** 399–408.
23. Kafri, T., van Praag, H., Gage, F. H., and Verma, I. M. (2000) Lentiviral vectors: regulated gene expression. *Mol. Ther.* **1,** 516–521.
24. Vigna, E., Cavalieri, S., Ailles, L., et al. (2002) Robust and efficient regulation of transgene expression in vivo by improved tetracycline-dependent lentiviral vectors. *Mol. Ther.* **5,** 252–261.
25. May, C., Rivella, S., Callegari, J., et al. (2000) Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. *Nature* **406,** 82–86.
26. May, C., Rivella, S., Chadburn, A., and Sadelain, M. (2002) Successful treatment of murine beta-thalassemia intermedia by transfer of the human beta-globin gene. *Blood* **99,** 1902–1908.
27. Lotti, F., Menguzzato, E., Rossi, C., et al. (2002) Transcriptional targeting of lentiviral vectors by long terminal repeat enhancer replacement. *J. Virol.* **76,** 3996–4007.
28. Zufferey, R., Donello, J. E., Trono, D., and Hope, T. J. (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73,** 2886–2892.
29. Klages, N., Zufferey, R., and Trono, D. (2000) A stable system for the high-titer production of multiply attenuated lentiviral vectors. *Mol. Ther.* **2,** 170–176.
30. Challita, P. M. and Kohn, D. B. (1994) Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. *Proc. Nat. Acad. Sci. USA* **91,** 2567–2571.
31. Reik, A., Telling, A., Zitnik, G., Cimbora, D., Epner, E., and Groudine, M. (1998) The locus control region is necessary for gene expression in the human β-globin locus but not the maintainance of an open chromatin structure in erythroid cells. *Mol. Cell. Biol.* **18,** 5992–6000.
32. Epner, E., Reik, A., Cimbora, D., et al. (1998) The β-globin LCR is not necessary for an open chromatin structure or developmentally regulated transcription of the native mouse β-globin locus. *Mol. Cell* **2,** 447–455.
33. Park, F. and Kay, M. A. (2001) Modified HIV-1 based lentiviral vectors have an effect on viral transduction efficiency and gene expression in vitro and in vivo. *Mol. Ther.* **4,** 164–173.

34. Dang, Q., Auten, J., and Plavec, I. (2000) Human beta interferon scaffold attachment region inhibits de novo methylation and confers long-term, copy number-dependent expression to a retroviral vector. *J. Virol.* **74,** 2671–2678.
35. Austin, T. W., Salimi, S., Veres, G., et al. (2000) Long-term multilineage expression in peripheral blood from a Moloney murine leukemia virus vector after serial transplantation of transduced bone marrow cells. *Blood* **95,** 829–836.
36. Agarwal, M., Austin, T. W., Morel, F., Chen, J., Bohnlein, E., and Plavec, I. (1998) Scaffold attachment region-mediated enhancement of retroviral vector expression in primary T cells. *J. Virol.* **72,** 3720–3728.
37. Orkin, S. H. (1995) Regulation of globin gene expression in erythroid cells. *Eur. J. Biochem.* **231,** 271–281.
38. Martin, D. I., Fiering, S., and Groudine, M. (1996) Regulation of beta-globin gene expression: straightening out the locus. *Curr. Opin. Gen. Dev.* **6,** 488–495.
39. Coffin, J. M., Huges, S. H., and Varmus, H. E. (1997) Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
40. Junker, U., Bohnlein, E., and Veres, G. (1995) Genetic instability of a MoMLV-based antisense double-copy retroviral vector designed for HIV-1 gene therapy. *Gene Ther.* **2,** 639–646.
41. Bordignon, C., Notarangelo, L. D., Nobili, N., et al. (1995) Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients. *Science* **270,** 470–475.

3

Choice and Use of Appropriate Packaging Cell Types

Annmarie L. Pacchia, Sayandip Mukherjee, and Joseph P. Dougherty

1. Introduction

Transducing lentiviral vectors that transfer the exogenous gene(s) into target cells are typically defective for viral replication because at least some of the *trans*-acting sequences encoding the viral proteins have been deleted. To propagate vector virus, the viral proteins are supplied either by transient transfection of plasmids expressing the viral proteins or by using packaging cell lines, which contain the viral expression plasmids stably integrated into their cell genome ***(1)***. Packaging cells provide a safety advantage for propagating retroviral vector virus because they reduce the chance of recombination that might result in the production of replication-competent (RC) virus. During development of the packaging cells, the expression plasmids can be introduced sequentially at different times so that they will be located at different places in the cell genome. In addition, avoiding cotransfection of all of the *trans*-acting components simultaneously, a recombinagenic step ***(2)***, further reduces the likelihood of recombination.

In the case of lentiviral vectors, particularly those derived from human immunodeficiency virus type 1 (HIV-1), additional safety elements are built into the system including dispensing with most of the genes encoding viral accessory and regulatory proteins while still retaining the ability of the vector to effectively transduce target cells. Moreover, to ensure safety, the packaging cells and their corresponding producer cells, which are packaging cells containing the transducing vector, are periodically monitored for the presence of RC virus.

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One difficulty with developing lentiviral packaging cells is that constitutive expression of some of the proteins can have cytotoxic or cytostatic effects on the cells. To overcome this difficulty, inducible expression systems, such as those based on tetracycline (tet) or ecdysone, can be used. The packaging cell lines are maintained in the absence of induction, and when vector virus production is required, they can be induced according to standard protocols.

There are at least three applications of lentiviral vectors and packaging cell systems: (1) for efficient gene transfer into dividing and nondividing cells, (2) for use in antiviral drug screening protocols, and (3) to study the basic biology of viral replication. The specific application dictates the type of packaging cell to be used. A packaging cell that expresses all of the lentiviral proteins might be more appropriate for drug screening or replication studies. A packaging cell that eliminates some of the viral genes for safety purposes is more appropriate for gene therapy approaches.

The focus of this chapter is to outline how to generate a stable, high-titer lentiviral vector-producing cell line as well as a sample of assays that can be used to characterize the cell line. The last section of the chapter briefly discusses important considerations when working with lentiviral vector-producing cells and the procedures commonly used to ensure the safety of laboratory personnel who use these cell lines and vector preparations.

2. Materials

1. Tissue culture treated dishes: 100 mm, 24- or 12-well plates, 6-well plates, and 96-well plates.
2. 293T cells, expression plasmids for the production of viral proteins, lentiviral vector or a 293T-based stable packaging cell line.
3. 10% MEM: minimal essential medium (MEM) containing Earle's salts and L-glutamine (GIBCO BRL [Carlsbad, CA] 11095-080) supplemented with 10% fetal bovine serum (FBS) (Hyclone [Logan, UT]), 0.2 m*M* MEM Nonessential Amino Acids Solution (GIBCO BRL 11140-050), 250 units/ml penicillin and 250 μg/mL streptomycin (GIBCO BRL 15070-083).
4. Trypsin solution (or other cell dissociation buffer).
5. Hemocytometer.
6. 5-mL polystyrene tubes, sterile (such as Falcon 2054, BD Biosciences, Bedford, MA).
7. 2.5 *M* calcium chloride, filter sterilized.
8. 2X BBS (BES-buffered solution): 50 m*M* N,N-bis (2-hydroxyethyl) (2-aminoethanesulfonic acid) (BES), 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4 (pH 6.95 with 1 *N* NaOH), H_2O to 1 L. Filter sterilized through a 0.45 μm nitrocellulose filter. Store in aliquots at –20°C.
9. Sterile, distilled water.
10. PBS (phosphate-buffered saline, pH 7.2) (GIBCO BRL 200012-027).

11. Antibiotic solution for selection of clones, filter sterilized.
12. Tweezers
13. Vacuum grease, autoclave sterilized (VWR 59340-000, Westchester, PA).
14. Raschig rings, 8 mm × 8 mm, autoclave sterilized (Sigma [St. Louis, MO] Z13759-6).
15. Ecdysone Induction System (Invitrogen K1001-01).
16. Ponasterone A (Invitrogen H101-01), resuspended in 100% ethanol to 1 m*M*. Store at –20°C after resuspension.
17. RT cocktail: 60 m*M* Tris-HCl (pH 7.8), 9 m*M* KCl, 6 m*M* $MgCl_2$, 0.06% Nonidet-P40, 6 μg/mL poly rA, 1.88 μg/mL oligo dT in water. Filter sterilize, aliquot, and store at –20°C.
18. 1 *M* dithiothreitol (DTT).
19. α^{32}P-TTP (10 μCi/μL).
20. Whatman DE81 filter paper.
21. 20X Saline sodium citrate: 175.3 g of sodium chloride plus 88.2 g of sodium citrate in 800 mL H_2O. Heat to 68°C to assist dissolution. Adjust to pH 7 by adding a few drops of conc. HCl. Adjust the volume to 1 L with H_2O and store at room temperature.
22. 95% ethanol.
23. 96-well replicator (Sigma Z37,081-9).
24. 0.45-μm syringe filters (with HT Tuffryn membrane; Pall Gelman, East Hills, NY).
25. (1 mg/mL) Polybrene. Polybrene solution made in PBS, filter sterilized and stored in aliquots at –20°C.

3. Methods

The methods below describe: (1) the development of a vector virus-producing cell line, (2) the induction and harvest of vector virus supernatants, (3) how to characterize the protein induction profile of the virus-producing cell line, and (4) safety considerations when working with lentiviral vector-producing cells.

3.1. Virus-Producing Cell Line

Developing a producer cell line involves the procedures outlined in **Subheadings 3.1.1.** through **3.1.3.** that include (1) a brief description of the expression plasmids required for virus production, (2) transfection of cells with these plasmids, and (3) selection of a cell line capable of high-titer vector virus production.

3.1.1. Protein Expression Plasmids and the Lentiviral Vector

The current generation of lentiviral vector systems typically consist of three to four different plasmids. Due to the cytotoxicity associated with high-level expression of some HIV-1 proteins, such as Env (envelope) and Protease and the vesicular stomatitis virus G glycoprotein (VSV-G) envelope protein, and

the cytostatic properties of HIV-1 Vpr, their production is usually controlled by the use of an inducible promoter *(3,4)*. The tetracycline inducible system has been used reliably by a number of researchers *(5,6)* while the ecdysone inducible system, though relatively new, is equally as efficient and easy to use for the production of viral proteins *(7)* (*see* **Note 1**). One of the first plasmids to be incorporated into the packaging system is that containing the transcriptional factors necessary for functionality of the inducible system **(Fig. 1)**. Second, the viral Gag and Pol proteins, as well as various HIV-1 accessory and regulatory proteins, are cloned into a plasmid under control of the inducible promoter. An alternative Gag-Pol expression vector for use in gene transfer studies may be designed to be devoid of any unnecessary accessory proteins, as shown in the Gag-Pol Vector II in **Fig. 1**. Third, the packaging cell will harbor another inducible expression construct containing the coding sequence for the viral envelope derived from HIV-1, VSV-G, or another virus of choice. These Gag-Pol and Env plasmids will provide all of the proteins that are necessary for virus production in *trans* without their genomes being packaged into the virions themselves since their mRNAs do not contain viral *cis* signals, such as the packaging (also known as encapsidation) signal. Finally, the producer cell line is established when a lentiviral vector construct is transfected into the packaging cells. In contrast to the viral protein expression plasmids, the lentiviral vector (also known as a transfer or transducing vector) contains all of the *cis*-acting signals for viral replication in addition to the exogenous gene(s) of interest that is driven by a heterologous promoter.

All of the cloning used to construct the Gag-Pol, Env, and transfer vectors is performed using standard recombinant DNA techniques *(8)*. After construction, each DNA is transformed into competent cells of the DH5α strain of *E. coli* and selected on LB (Luria-Bertani Broth, Miller) plates containing ampicillin (100 μg/mL). Bacterial colonies are grown up, confirmed by miniprep analysis, and finally, amplified and purified in large scale by utilizing a cesium chloride density gradient protocol *(8)*.

3.1.2. Transfection

There are two different schemes for the production of lentiviral vectors from cells. The first type of production system for lentiviral vectors is highly reproducible, has a much lower chance of recombination, and therefore, a great degree of safety. It involves transfecting a stable, inducible packaging cell line with a single plasmid containing the lentiviral vector, followed by subsequent selection for that vector (outlined in **Subheading 3.1.3.**) and the establishment of a stable, inducible virus-producing cell line.

For the stable transfection of 293T cells or 293T-based lines, a modified calcium phosphate precipitation protocol yields favorable results.

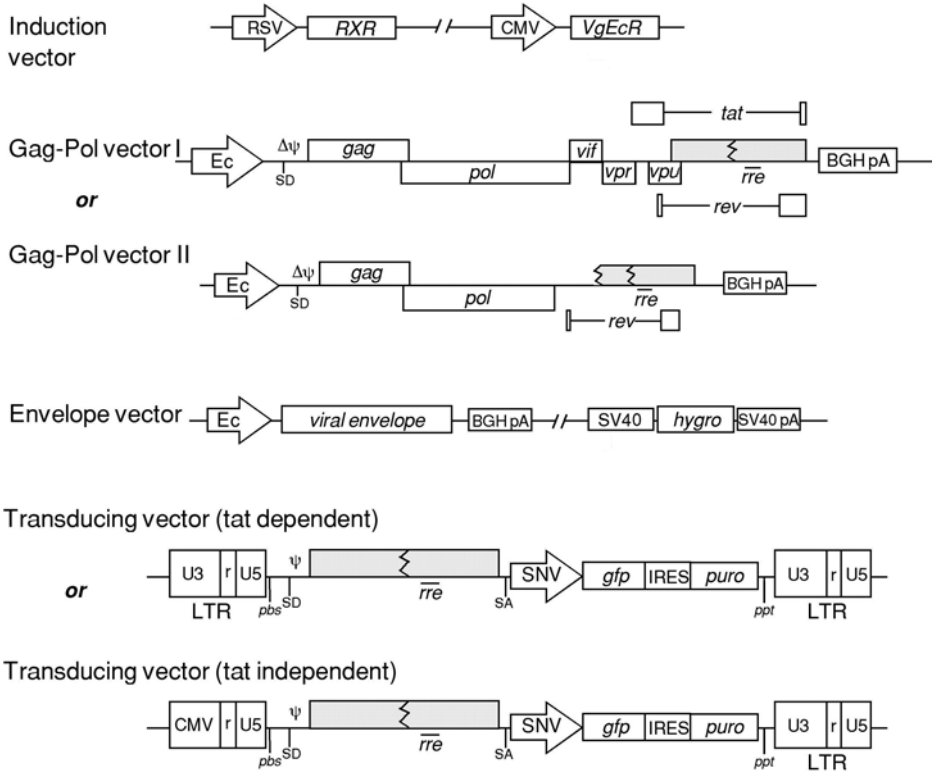

Fig. 1. Schematic diagrams of the expression plasmids and retroviral transducing vector that can be used to establish a stable, inducible lentiviral vector producer cell line. The Induction vector encodes the subunits of the transcriptional heterodimer for the inducible ecdysone system. Gag-Pol vector I is an example of a packaging construct that encodes Gag, Pol, and all of the accessory genes. Gag-Pol vector II is a second type of packaging construct that also encodes Gag, Pol, and one regulatory gene, *rev*, but lacks *tat* and all of the accessory genes. The Envelope vector encodes the viral envelope, the most common being HIV-1 Envelope or VSV-G. The Transducing vector encodes the gene of interest (*gfp* in this case) driven from an internal promoter. It may be tat-dependent or tat-independent depending upon whether its being used with Gag-Pol vector I or II respectively. Boxes shaded with diagonal lines depict nonfunctional HIV-1 genes. Abbreviations and symbols: ΔΨ represents a 33-bp deletion within the encapsidation signal downstream of the splice donor site. RSV, Rous sarcoma virus promoter; RXR, Retinoid X receptor; CMV, immediate-early promoter from human cytomegalovirus; VgEcR, modified ecdysone receptor; Ec, inducible ecdysone promoter; SD, splice donor site; IRES, internal ribosomal entry site from encephalomyocarditis virus; *gfp*, sequence encoding the green fluorescent protein; BGH pA, bovine growth hormone polyadenylation signal; SV40, SV40 early gene promoter; *hygro*, hygromycin resistance gene; SV40 pA, SV40 polyadelylation signal; SNV, spleen necrosis virus U3 promoter; LTR, HIV-1 long terminal repeat; *puro*, puromycin resistance gene.

1. Approximately 24 h before the transfection, a confluent plate of 293T-based packaging cells is trypsinized, and the cells are resuspended in MEM supplemented with 10% fetal calf serum (FCS). A small aliquot is used for counting with a hemocytometer. Once the concentration of cells is known, 2.0×10^6 cells are plated in a 100-mm tissue culture dish in 10% MEM in a final volume of 9 mL. The plated cells are kept in a 5% CO_2 atmosphere at 37°C prior to transfection.
2. For the establishment of stable cell lines, use approximately 10 µg of the transfer vector DNA per plate to be transfected.
3. Dilute a 2.5 *M* stock of calcium chloride 10-fold with sterile, distilled water to 0.25 *M*, allowing for 0.5 mL of 0.25 *M* calcium chloride per plate.
4. In a sterile 5-mL polystyrene tube, dispense 0.5 mL of 0.25 *M* calcium chloride. Use one tube for each transfection.
5. Add the DNA to be transfected into the tube containing the calcium chloride. Mix gently by tapping the tube.
6. Slowly add 0.5 mL of 2× BBS to the tube, allowing some air to bubble through the solution (using the pipet) after dispensing in order to very gently mix the components.
7. Allow the tube to sit at room temperature for 15 to 20 min. A very fine, white precipitate will begin to form.
8. Pipet the 1-mL calcium/DNA/BBS solution dropwise onto the plate of 293T cells that were seeded in 9 mL of 10% MEM the previous day. As the solution is added, swirl the dish gently in order to mix.
9. Incubate the transfections in an incubator at 3% CO_2 atmosphere and 35°C overnight (16–20 h).
10. To terminate the transfection, wash each plate two times with 8 mL of prewarmed PBS, gently swirling the dish to facilitate the wash.
11. After the last wash with PBS, refeed the transfected cells with 7–10 mL of 10% MEM.
12. Twenty-four hours after terminating the transfection, 10% MEM containing an antibiotic for selection of the transfer vector is added to the cells (*see* **Note 2**).

3.1.3. Isolating a Stable, High-Titer, Vector-Producing Cell Line

After following the protocol for acquiring stable transfectants, when antibiotic selection is complete, small cell clones will begin to grow on the transfected plate. These clones are small groups of cells that have grown up from a single transfected, antibiotic-resistant cell and represent the putative lentiviral producer cell lines. To isolate these individual clones for further expansion, it is best to wait until they are a sufficient size to allow the cells to survive on a larger surface area. This may take anywhere from 1 to 3 wk after selection to occur.

1. With the help of a low power compound microscope, circle each clone to be isolated with a marker on the bottom of the dish.

2. Aspirate the media from the transfected plate containing the cell clones and wash with 8 mL PBS to remove traces of the media and selective antibiotics.
3. After removing the PBS wash, pipet approx 1 mL of fresh PBS onto the dish to keep the cells wet.
4. Using sterilized tweezers, pick up a sterile, glass Raschig ring by one open end. Inspect it for any chips or breaks and discard rings that have these defects (*see* **Note 3**).
5. Place the other open end of the ring into the sterile vacuum grease to coat the edge of the ring (*see* **Note 4**).
6. Place the greased ring around each circled clone on the plate. The grease helps to form a seal between the ring and the plate so that when trypsin is added, it will only lift the cells within the ring.
7. Repeat **steps 4–6** until all clones of interest are encircled with a ring.
8. Using a small serological pipet, add trypsin to fill each ring.
9. Make ready a well plate with fresh 10% MEM with selection for plating the newly isolated clones. Depending on the size of the clone, wells as small as those on a 96-well plate can be used. However, if the clone is of substantial size or the cell type grows quickly, choose a plate with a larger surface area, such as a 48- or 24-well plate.
10. Use a pipet outfitted with a 200 μL-sized tip to pipet the trypsin up and down in the ring to break up the clone and make an even cell suspension.
11. Place the cells into an individual well containing selection media.
12. Repeat **steps 10** and **11** until all clones are picked up from the dish. Be sure to use a fresh pipet tip for each clone in order to prevent cross-contamination.

As the producer cell clones begin to grow to confluency in the wells, they should be trypsinized and placed into a larger dish until they are of a sufficient quantity to allow for archiving by freezing. From this point on, maintain and passage each clone according to standard tissue culture practices.

3.2. Production and Harvest of Vector Virus

Once the putative producer line is selected, expanded, and archived, the next steps involve the actual induction and collection of virus supernatant for subsequent determination of vector virus titer. **Subheading 3.2.** outlines how to use an inducible system for the production of vector virus (using the ecdysone inducible system as an example) and determine the best time to harvest viral supernatants from the induced producer cell line.

3.2.1. The Ecdysone Inducible System and Production of Vector Virus

The ecdysone system is an inducible gene expression system for mammalian cells that is predicated on the ability of an analog of ecdysone, ponasterone A, to activate transcription through the binding of a heterodimer consisting of a

modified ecdysone receptor (VgEcR) and its binding partner, the retinoid X receptor (RXR) **(Fig. 1)** *(7)*. The VgEcR protein has been modified to contain the VP16 transactivation domain from herpes simplex virus that allows for gene expression in mammalian cells. Addition of ponasterone A to the cells causes the VgEcR/RXR heterodimer to bind to a hybrid response element and to activate gene transcription from a proximal heat shock promoter *(7)*.

Establishing a lentiviral producer cell line under control of the ecdysone system allows for rapid and high-level expression of all of the proteins necessary for the efficient production of vector virus stocks *(9,10)*. The protocol that follows details the induction process for one type of stable, 293T-based producer cell line. These parameters should be used as a guideline or starting point since the most appropriate seeding density as well as the concentration and timing (i.e., single or double induction) of the addition of ponasterone A should be optimized for each cell line.

1. Twenty-four hours prior to induction, plate 2.5×10^6 293T-based lentiviral producer cells in a 100-mm dish in 10% MEM without selective antibiotics.
2. Forty-eight hours after seeding, replace the 10% MEM with 8–10 mL fresh 10% MEM containing 10 μ*M* ponasterone A (*see* **Note 5**).
3. Forty-eight hours after the first induction, replace the media with another 8–10 mL 10% MEM containing 10 μ*M* ponasterone A.
4. Supernatant containing vector virus is typically harvested 48 h after the second induction for the lines that we have developed. However, it is a good idea to determine the optimal time for virus isolation with new cell clones as described below.

3.2.2. Determining the Optimal Time for Harvesting Vector Virus from a Lentiviral Producer Cell Line

Regardless of the type of inducible system, the optimal time of viral harvest after induction needs to be determined empirically in order to achieve the highest possible titer.

1. Twenty-four hours prior to induction, set up a series of plates seeded with the appropriate number of producer cells. The series should include one plate of producer cells that will remain uninduced as a control and one plate for each day that supernatant will be harvested postinduction.
2. The following day, induce all of the experimental plates using the optimized amount and timing of inducer which was determined in the experiment described in **Subheading 3.2.1.** Be sure to keep the timing and amount of inducer the same for every plate in the series.
3. Beginning 24 h after the induction, harvest viral supernatant from the first plate for use in a viral titration assay. Cells for the virus titration should have been seeded the night before the infection. (*see* **Subheading 3.3.2.** and Chapter 5 for

details and protocols). Also, be sure to collect supernatant from the uninduced control plate and set up a viral titration of this sample as well.
4. Continue harvesting and titrating viral supernatants every 24 h from the remaining plates.
5. Calculate the viral titer from each experiment and use this information to determine the optimal time of exposure to the inducer and, therefore, the best time to harvest.

3.3. Characterizing the Producer Cell Line

It is often useful to characterize an inducible lentiviral vector producing cell line by assessing the relative levels of protein production from the plasmids stably integrated within the cells and evaluating the efficiency of virus production. Some standard protocols already exist for the former: Western blots can be used to investigate the expression of the viral envelope protein since many different envelope antisera are readily available, and commercially available kits are utilized to perform enzyme-linked immunosorbent assays (ELISA) for the quantitation of the HIV capsid protein, p24. As an alternative, this section of the chapter details assays for detecting reverse transcriptase (RT) and quantitating viral titer.

3.3.1. HIV-1 Reverse Transcriptase Assay

The induction process can be observed by analyzing the relative amount of HIV-1 reverse transcriptase production using the following assay.

1. Prior to the experiment, collect supernatant samples from uninduced and induced producer cells and store them at –20°C.
2. At the time of the RT assay, thaw the supernatant samples (e.g., d 0 to d 7 samples) to be tested and place 10 µL duplicate samples into the wells of a 96-well plate. Be sure to add negative control wells that consist of media only.
3. Thaw enough RT cocktail to allow for approx 50 µL of cocktail per well.
4. To the RT cocktail add 1 *M* dithiothreitol (DTT) to 2.4 m*M* and 0.5 µCi of α^{32}P-TTP (this is approx 1 µL of 10µCi/µL α^{32}P-TTP per ml of RT cocktail). Mix well.
5. Add 50 µL of the RT cocktail to each well containing 10 µL of supernatant from the producer cells. Mix by shaking gently.
6. Incubate the 96-well plate at 37°C for 90 min.
7. Cut two pieces of DE81 Whatman paper to the approximate size of the 96-well plate, allowing for some excess. Pin each piece down onto a styrofoam support covered with plastic wrap.
8. Spot the samples onto the first piece of DE81 Whatman paper. The spotting can be done with a disposable 96-well replicator or with a reusable metal-pronged replicator. The volume is approx 10 µL/spot.

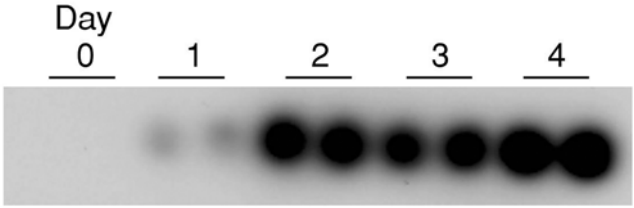

Fig. 2. Autoradiographic results of an assay to detect reverse transcriptase. Supernatant samples from an ecdysone inducible lentiviral producer cell line were collected prior to induction (Day 0) and on Days 1 through 4 postinduction and assayed as described in the text.

9. Replicate to the second sheet of DE81 two times. Try to place the second replication on top of the first.
10. Let the filters air dry at room temperature.
11. Wash the blots with 2 × SSC three times, 10 min each, on a slow shaker at room temperature.
12. Wash blots with 95% ethanol for 5 min at room temperature.
13. Allow the blots to air dry at room temperature.
14. Cover the radioactive blots with plastic wrap and either expose to film at –70°C overnight or to an imaging screen for analysis. *See* **Fig. 2** for an example of results from a reverse transcriptase assay.

3.3.2. Vector Virus Titration

There are numerous protocols in current use for determining the titer of vector virus produced from a lentiviral producer cell line. Given below is one example of a virus titration procedure.

1. In a 6-well plate, seed 2.5×10^5 target cells (*see* **Note 6**) in 10% MEM 18–24 h before infection. Plate a sufficient number of wells in order to perform the infections at two or three different dilutions in duplicate.
2. On the day of infection, harvest vector virus supernatant from the lentiviral producer cell lines (time of harvest is determined empirically as described in **Subheading 3.2.2.**). Filter the supernatant through a 0.45 μ*M* pore-size syringe filter to remove any cellular debris.
3. Make serial dilutions of the harvested virus into 10% MEM containing 8 μg/mL polybrene. Note that if the volume of supernatant added to the first dilution is greater than 10% of the final volume, be sure to adjust the concentration of polybrene to 8 μg/mL before continuing with the dilutions. If no dilutions are to be made, add polybrene to the undiluted viral supernatant to 8 μg/mL.
4. Aspirate the media from the seeded target cells. Place 1 mL of each supernatant into its corresponding well in the 6-well plate.
5. Incubate the transduction for 6–8 h at 37°C, 5% CO_2.

6. To terminate the infection, aspirate the virus from each well, being careful not to cross-contaminate wells with different concentrations of virus. Replace the viral media with 10% MEM.
7. If the transducing vector harbors a gene for antibiotic selection, the selective media can be added to the cells 24 h postinfection and be refreshed every 3–4 d until transduced target cell clones are apparent.
8. Using this method of transduction and selection, the number of resistant clones can be used to calculate the viral titer as follows: the number of antibiotic-resistant colonies is divided by the volume of the inoculum (in milliliters) and the result is multiplied by the dilution factor. Viral titer is then expressed as infectious units per milliliter.

3.4. Considerations

When working with lentiviral vector systems, certain precautions should be taken into consideration. Outlined here are National Institutes of Health-Centers for Disease Control (NIH-CDC) guidelines for the handling of recombinant DNA and the cells and viruses containing recombinant DNA.

3.4.1. Safety Guidelines

Briefly, the following policies for working with lentiviral vectors should be instituted:

1. Access to the laboratory should be limited or controlled as determined by the principal investigator.
2. All laboratory and at-risk personnel should be subjected to a blood test prior to granting access to the laboratory and periodically thereafter.
3. Display biohazard warning signs on the access door to the laboratory.
4. Wear protective lab clothing including lab coats and gloves.
5. Decontaminate all waste generated in the laboratory by autoclave sterilization before disposal.
6. An open bench and a biosafety cabinet/hood should be readily available.
7. Only mechanical pipetting devices should be used; mouth-pipetting is strictly prohibited.
8. Use of "Sharps" is not allowed (or is highly discouraged). Appropriate precautions should be taken for any glass slides that may be used in the laboratory.

More detailed information is available in the CDC-NIH Biosafety in Microbiological and Biomedical Laboratories, *HHS Publication No. 93-8395* and NIH Guidelines for Research involving Recombinant DNA.

4. Notes

1. The tetracycline inducible systems fall into two broad categories: the conventional *tet*-off system and the reverse-*tet*, or *tet*-on, system. In the *tet*-off system, the continued presence of tetracycline is required to maintain the repressive or

"off" state. Inconvenience notwithstanding, it has been reported that constitutive expression of the transactivation protein tTA can be cytotoxic ***(11,12)***. A system in which transcription is activated by drug addition may serve as a more approachable, useful alternative. Toward this end, the *tet*-on system utilizes a mutant tet receptor that suppresses gene expression in the absence of the inducer and allows expression to occur upon addition of tetracycline or its analog doxycycline ***(13)***. Although it is less cumbersome than the *tet*-off system, some noted drawbacks include significant basal expression in the absence of the inducer and inconsistent gene expression ***(12)***. The ecdysone system, which is also easy to manipulate, remedies the issue having to do with background expression. The transcriptional activation components, RXR and VgEcR, can only bind to the response element containing the promoter in the presence of the inducer ponasterone A. In addition, they are typically not cytotoxic, even when constitutively expressed at high levels in the host cells. Overall, the ecdysone system provides for low to zero background in the absence of the inducer, and once ponasterone A is added, it triggers a very robust induction of the target gene with no deleterious effects occurring in the cells that are treated with it ***(7,9,10)***.

2. Selection media helps to isolate cells which have incorporated the vector plasmid by selecting for an antibiotic resistance marker contained within the body of the vector that has just been introduced to the cells. Depending on the selectable marker used, the selection process can take from 4 d to 2 wk. The concentration of antibiotic that is used should be previously determined by performing a "kill curve" on the packaging cell line using varying concentrations of the drug in order to find an optimal concentration. Generally, this concentration will be enough to kill 100% of nontransfected cells during a selection period of 5–14 d.
3. The size of the Raschig ring given in this protocol is 8 mm × 8 mm, which is the most versatile for picking up clones of various sizes. If the cell clones to be isolated are or need to be much smaller in size, Raschig rings with a smaller diameter are available and can be used after sterilization using the same approach as described in the text.
4. To sterilize the Raschig rings and the vacuum grease, use Pyrex or Kimax brand glass petri dishes with covers which can withstand repeated autoclaving. A 100- × 15-mm dish is sufficient for the Raschig rings, while a smaller 60- × 15-mm dish is more appropriate for the grease. Fill the larger dish with rings so that the cover still fits. Into the smaller dish, scoop out enough grease from its original container to thickly coat the bottom of the glass petri dish. Top each dish with its cover and a piece of autoclave tape and set to sterilize in a dry cycle according to your standard procedure.
5. It is important to note that, in our experience, 293T cells and 293T-based producer cells tend to demonstrate reduced adherence on induction with ponasterone A. Therefore, extreme care should be taken when inducing and reinducing the producer cells in order to avoid having the cells completely "lift" from the surface of the plate. Their dissociation can result in a reduction in viral titer and, if extensive, may make filtration of the supernatant prior to infection problematic.

If reduced adherence is a consistent issue, a different seeding density or induction scheme may help to alleviate, at least partially, the problem.

6. Target cells for an infection protocol should be chosen according to the tropism of the virus produced from the producer cell line. For example, if the virus produced is pseudotyped with the VSV-G envelope protein, many different mammalian cell types can be used as targets, including HeLa, HeLaT4, 293T, and HOS. However, if the envelope provided by the packaging cell is HIV-1-based, only $CD4^+$ cells will be susceptible to infection. Furthermore, it is important to note the coreceptor tropism of the HIV-1 envelope which will be used because cell surface coreceptor usage varies depending on the strain of HIV-1 used to infect the target cells.

References

1. Buchschacher, G. L., Jr. and Wong-Staal, F. (2000) Development of lentiviral vectors for gene therapy for human diseases. *Blood* **95,** 2499–2504.
2. Mann, R., Mulligan, R. C., and Baltimore, D. (1983) Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33,** 153–159.
3. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J. K. (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* **90,** 8033–8037.
4. Kaplan, A. H. and Swanstrom, R. (1991) The HIV-1 gag precursor is processed via two pathways: implications for cytotoxicity. *Biomed. Biochim. Acta* **50,** 647–653.
5. Yu, H., Rabson, A. B., Kaul, M., Ron, Y., and Dougherty, J. P. (1996) Inducible human immunodeficiency virus type 1 packaging cell lines. *J. Virol.* **70,** 4530–4537.
6. Kafri, T., van Praag, H., Gage, F. H., and Verma, I. M. (2000) Lentiviral vectors: regulated gene expression. *Mol. Ther.* **1,** 516–521.
7. No, D., Yao, T. P., and Evans, R. M. (1996) Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* **93,** 3346–3351.
8. Ausubel, F. M., Brent, R., Kingston, R. E., et al., eds. (1994) *Current Protocols in Molecular Biology.* Wiley, Hoboken, NJ.
9. Pacchia, A. L., Adelson, M. E., Kaul, M., Ron, Y., and Dougherty, J. P. (2001) An inducible packaging cell system for safe, efficient lentiviral vector production in the absence of HIV-1 accessory proteins. *Virology* **282,** 77–86.
10. Sparacio, S., Pfeiffer, T., Schaal, H., and Bosch, V. (2001) Generation of a flexible cell line with regulatable, high-level expression of HIV Gag/Pol particles capable of packaging HIV-derived vectors. *Mol. Ther.* **3,** 602–612.
11. Shockett, P., Difilippantonio, M., Hellman, N., and Schatz, D. G. (1995) A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* **92,** 6522–6526.

12. Saez, E., No, D., West, A., and Evans, R. M. (1997) Inducible gene expression in mammalian cells and transgenic mice. *Curr. Opin. Biotechnol*. **8,** 608–616.
13. Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science* **268,** 1766–1769.

4

Small- to Large-Scale Production of Lentivirus Vectors

Michael P. Marino, Milson J. Luce, and Jakob Reiser

1. Introduction

1.1. Production of Lentiviral Vectors by Transient Transfection

Lentiviral vectors have traditionally been produced in human embryonic kidney 293T cells ***(1)***, involving transient transfection procedures that were originally established for the production of retroviral vectors based on Moloney murine leukemia (MoMLV) virus ***(2)***. Simian virus 40 (SV40)-transformed African green monkey kidney (COS-7) cells ***(3)*** and human TE671 rhabdomyosarcoma cells ***(4)*** have also been used to generate human immunodeficiency virus type 1 (HIV-1)-based lentiviral vectors ***(5–7)***, but titers obtained in 293T cells are generally higher than those observed in other cell lines. Page *et al.* ***(5)*** were the first to describe an HIV-1-based vector harboring a selectable transgene. The HIV-1 genome was rendered replication defective in this study by replacing the envelope (Env)-encoding gp160 sequence with a guanine-phosphoribosyl transferase *(gpt)* gene driven by the SV40 early promoter. Transient cotransfection of COS-7 cells with this *env*-deleted vector and a gp160 expression vector resulted in packaging of the defective HIV-gpt genome into infectious virions. Upon infection of susceptible cells, the *gpt* drug resistance gene was transmitted and expressed, allowing transduced cells to be selected in mycophenolic acid ***(5)***. Landau *et al.* ***(8)*** subsequently demonstrated that expression of heterologous Env proteins, including the MoMLV Env and the human T-cell leukemia virus type I (HTLV-I) Env, in cells transfected with the HIV-gpt vector resulted in the production of vector pseudotypes capable of infecting human as well as murine cells with titers reaching 10^5 colony-forming units (CFU) per mL. These observations were confirmed and extended

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in 1996 by results that showed that the vesicular stomatitis virus G glycoprotein (VSV-G) was also efficiently incorporated into HIV-1 virions *(6,9,10)*, yielding titers ranging from 10^5 transducing units (TU) per mL to 10^7 CFU per mL and above *(6)*. The transient vector production system has since been optimized and HIV-1 vectors pseudotyped with VSV-G at titers well above 10^7 TU/mL are now routinely obtained *(11–13)*.

1.2. Production of Lentiviral Vectors Using Packaging Cell Lines

Large-scale preclinical applications in animals and clinical trials in humans make the availability of high-titer lentiviral packaging cell lines for vector production very desirable. Initially, such cell lines performed poorly, and the resulting vector titers were low *(14,15)*. A major difficulty in the construction of packaging cell lines for lentiviral vectors is that some of the HIV-1 proteins including protease are cytotoxic *(16)*, whereas others like Vpr have been shown to be cytostatic *(17)*. Moreover, VSV-G, the most commonly used Env in producing lentiviral pseudotypes, is highly fusogenic *(18)* and ultimately cytotoxic. Improved packaging cell lines containing regulatable expression cassettes based on the tetracycline (Tet)-regulatable transactivator system *(19)* or the ecdysone-inducible system *(20)* were subsequently established *(21–28)*. Regulated expression of protease and VSV-G allowed the generation of stable cell lines providing viral titers up to 10^6 TU per mL. Although these titers still fall short of the highest yields obtained by transient transfection, such virus stocks could nonetheless be concentrated by ultracentrifugation to titers reaching 10^9 TU/mL *(23,24,27)*. Advantages of stable cell lines over transient transfection systems include enhanced reproducibility and scalability of the production process and improved safety.

1.3. Lentiviral Pseudotypes

HIV-1 has long been known to form pseudotypes by the incorporation of heterologous glycoproteins through phenotypic mixing, allowing an extension of the host range of such pseudotypes beyond cells expressing the CD4 receptor. Several studies have demonstrated that wild-type HIV-1 produced in cells infected with xenotropic murine leukemia virus *(29,30)*, amphotropic murine leukemia virus *(31,32)*, or herpes simplex virus *(33)* gave rise to phenotypically mixed virions with an expanded host range, suggesting that pseudotyped virions were produced. Phenotypic mixing of viral Env proteins has also been shown to occur between HIV-1 and VSV in coinfected cell cultures *(33)*.

Besides VSV-G and the MoMLV-derived ecotropic and amphotropic Env glycoproteins *(6,10,34)* and the HTLV-I Env *(8,35)*, a growing list of glycoproteins derived from a variety of different enveloped viruses has been described that form stable pseudotypes with HIV-1 vector cores in vitro. These include

the gibbon ape leukemia virus (GALV) Env ***(36,37)***; the Env proteins of the MoMLV 10A1, polytropic, and xenotropic subtypes ***(36,37)***; the avian leukosis virus subtype A Env ***(38)*** (Zhang, X.-Y. and Reiser, J., unpublished), and the RD114 Env of the cat endogenous retrovirus ***(13)*** (Zhang, X.-Y. and Reiser, J., unpublished). Nonretrovirus-derived glycoproteins were also tested, including the rabies G glycoprotein ***(11)***, the G glycoprotein of a related lyssavirus, Mokola virus ***(11)***, the lymphocytic choriomeningitis virus (LCMV) glycoprotein ***(37–39)***, the hemagglutinin protein of influenza virus ***(37–40)***, the respiratory syncytial virus (RSV) F and G surface glycoproteins ***(40)***, and the Ebola virus and Marburg virus glycoproteins ***(40,41)***. These alternative pseudotypes may ultimately help bypass toxicity problems that are inherent to VSV-G ***(39)***, although the titers obtained with these alternatives are at present generally lower than those observed with VSV-G ***(11)***.

1.4. Methods to Concentrate and Purify Lentiviral Vectors

Typical lentiviral titers obtained by transient transfection range from 10^6 to 10^7 TU per mL. Increased titers can be achieved by such physical concentration as ultracentrifugation and ultrafiltration of virus-containing cell culture supernatants. Using a protocol initially designed for the generation of high-titer MoMLV vector stocks ***(42)***, pseudotyped lentiviral particles containing the VSV-G glycoprotein have been shown to be stable and to withstand concentration by ultracentrifugation without significant loss in titer ***(10,43)***. This has facilitated the generation of highly concentrated vector stocks for in vivo applications. HIV-1 pseudotypes containing other Env glycoproteins can also be concentrated by ultracentrifugation. Reiser ***(44)*** showed that HIV-1 vector cores harboring the MoMLV 4070A Env or the rabies virus G glycoprotein were concentrated effectively, with recovery of 80% or more of infectious particles. HIV-1 pseudotypes harboring a variety of different Env glycoproteins have since been shown to lend themselves to concentration by ultracentrifugation. These proteins include the avian leukosis virus subtype A Env ***(38)*** (Zhang, X.-Y. and Reiser, J., unpublished), the Mokola virus G glycoprotein, the glycoproteins of the Zaire and Reston subtypes of the Ebola virus, the influenza virus hemagglulinin (HA) protein, the RSV F and G surface glycoproteins ***(40)***, and the RD114 Env ***(13)*** (Zhang, X.-Y. and Reiser, J., unpublished).

Low-speed centrifugation approaches have also been used for concentrating lentiviral vectors including HIV-1-based vectors ***(45)*** and feline immunodeficiency virus (FIV)-based vectors ***(46)***. Concentration by low-speed centrifugation overnight resulted in virus preparations with yields of infectious particles up to 70% ***(45)***.

Concentration protocols based on ultrafiltration were also established for HIV-1-based vectors. Reiser et al. ***(6)*** have developed a facile ultrafiltration

procedure based on Centriprep units (Millipore, Bedford, MA) to concentrate HIV-1 pseudotypes. Good recoveries were observed with HIV-1 particles containing VSV-G or the MoMLV amphotropic Env. A scaled-up ultrafiltration procedure based on Centricon Plus-80 units, manufactured by Millipore, was subsequently reported ***(44)***. This system was found to be effective at concentrating HIV-1 particles displaying VSV-G, the MoMLV 4070A, or the rabies virus G glycoprotein, with volume reductions up to 100-fold and recoveries up to 100% and above. Yields greater than 100% are presumably achieved by removal of as yet unidentified inhibitors of viral infection from the preparation. Vector stocks concentrated on Centricon Plus-80 units (Millipore) were found to be efficient at transducing rat cerebellar neurons ***(47)*** and mouse and human hematopoietic progenitor cells ***(13,48)***.

Vector production for large-scale in vivo applications that require high-titer stocks is challenging due to the lack of simple procedures capable of rapidly processing large volumes of cell culture supernatant. The traditional ultracentrifugation-based approaches are limited in terms of their capacity to handle large volumes, thus making this procedure extremely tedious. Centrifugation at low speed overnight has been used to process volumes in excess of 1L following virus production using Nunc Cell Factories (Nalge Nunc, Naperville, IL) ***(45)***. However, this low-speed approach is time consuming.

There is an emerging need for quick, reproducible, and less laborious procedures that rapidly reduce the volume of the cell culture supernatant to be processed. Pham et al. ***(49)*** have described a gentle and simple precipitation method that rapidly concentrates both MoMLV and lentiviral vectors involving the coprecipitation of pH-adjusted viral supernatants with calcium phosphate, low-speed centrifugation, and dialysis. Volumes were decreased from 300 mL to 10 mL, with 50–100% recovery of infectious virus. Zhang et al. ***(50)*** have presented an alternative precipitation method based on poly-L-lysine (PLL). Viral supernatants were mixed with PLL (0.005% final concentration), incubated for 30 min at 4°C, and then centrifuged at 10,000*g* for 2 h. Recovery of infectious virus ranged from 26 to 32% after processing up to 3 L cell culture supernatant.

One problem with the methods just outlined is that cell-derived components are concentrated along with the vector particles. Thus, ultracentrifugation/ultrafiltration and precipitation approaches generate rather impure virus preparations and additional steps are required in order to purify the virus from contaminating host cell components. A method based on anion exchange chromatography of lentiviral supernatants on a Fractoflow 80-6 column (Merck KGaA, Darmstadt, Germany) was recently described ***(51)***. Viral particles bound to the column were eluted at 2 *M* NaCl in phosphate-buffered saline (PBS), desalted, and concentrated by centrifugation using 100,000 MW cutoff

Vivaspin (Vivascience, Hannover, Germany) filters. Yields and purity of the virus stocks resulting from this procedure were not reported, but this approach may represent one method of generating vector stocks with improved purity and reduced toxicity.

2. Materials

2.1. Cells

1. 293T cells ***(1)***.
2. 293 ALF10 cells ***(44)***. Based on 293 cells (ATCC, CRL-1573) that were stably transfected using the pALF plasmid ***(52)*** to express the amphotropic 4070A Env.

2.2. Cell Culture

1. Dulbecco's modified Eagle's medium (DMEM)/10% FBS (fetal bovine serum): The following are added directly to DMEM (high glucose) (GIBCO, Carlsbad, CA) to give the indicated final concentrations: 10% heat-inactivated (56°C for 30 min) fetal bovine serum (Hyclone, Logan, UT), 50 U/mL penicillin (GIBCO), 50 μg/mL streptomycin (GIBCO), 2 m*M* L-glutamine (BioWhittaker, Walkersville, MD). The final medium is filtered through a 0.22-μm filter, stored at 4°C, and used within 1–2 wk.
2. UltraCULTURE: The following are added to UltraCULTURE (BioWhittaker) to give the indicated final concentrations: 50 U/mL penicillin, 50 μg/mL streptomycin, 2 m*M* L-glutamine. The final medium is filtered through a 0.22-μm filter, stored at 4°C, and used within 1–2 wk.
3. Freezing medium (GIBCO) is stored at –20°C.
4. Zeocin (100 mg/mL, Invitrogen, Carlsbad, CA) is stored at –20°C. 293 ALF10 cells are grown in DMEM/10% FBS/Zeocin (125 μg/mL).
5. Chloroquine. The chloroquine (Sigma, St. Louis, MO) stock concentration is 25 m*M* (dissolved in PBS and subsequently filtered through a 0.45-μm filter and stored at –20°C).

2.3. Virus Production

1. 2X HEPES-buffered saline (HBS): 50 m*M* N-2-hydroxyethylpiperozine-N-ethanesulfonic acid (HEPES) (GIBCO), 10 m*M* KCl, 12 m*M* dextrose (D-glucose), 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4. The final pH of the solution should be 7.05 ± 0.05. The solution is filtered through a 0.45-μm filter, aliquoted, and stored at –20°C.
2. 2 *M* $CaCl_2$: a 2 *M* solution of $CaCl_2$ (Sigma) is prepared in H_2O, filtered through a 0.45-μm filter, aliquoted, and stored at –20°C.
3. Polyethylene glycol (PEG): a 50% PEG 6000 (Fluka) solution is prepared in H_2O, autoclaved, and stored at 4°C.
4. NaCl: a 4 *M* NaCl solution is prepared in H_2O, autoclaved, and stored at 4°C.
5. Plasmid DNA is purified using QIAfilter plasmid isolation kits (Qiagen, Valencia, CA).

3. Methods (*see* Note 1)

3.1. Small-Scale Transfection (see Note 2)

All conditions described are for a single well of a 6-well plate.

1. Plate 6×10^5 cells/well in 2 mL of DMEM/10% FBS 24 h prior to transfection. The well should be no more than 50% confluent at the time of transfection.
2. Just prior to transfection, add chloroquine to give a final concentration of 25 μ*M* (*see* **Note 3**).
3. Prepare the transfection mixture in a 5-mL (12 × 75 mm) polystyrene tube by adding 4.8 μg vector DNA, 3.2 μg helper DNA, and 1.6 μg Env plasmid DNA (*see* **Note 4**) in a total of 219 μL H_2O. Add 31 μL 2 *M* $CaCl_2$.
4. Add 250 μL 2X HBS to the transfection mixture while vortexing the tube (*see* **Note 5**). Immediately (within 1 to 2 min) add this solution to the cells and gently agitate the plate to ensure uniform mixing. Return cells to the incubator.
5. Change medium 12–16 h after transfection (*see* **Note 6**).
6. Harvest the lentiviral supernatant 60 to 65 h posttransfection by gently removing the supernatant and filtering it through a 0.45-μm filter (*see* **Note 7**). Aliquot the filtrate into cryotubes.
7. To freeze the lentiviral supernatant, place vials on dry ice for 10 min, then transfer the frozen samples to –80°C for storage (*see* **Note 8**).

3.2. Large-Scale Transfection

All conditions are described for 150-mm culture dishes (*see* **Note 9**).

1. Plate 8×10^6 293T cells per 150 mm culture dish approximately 18–24 h prior to transfection in 25 mL DMEM/10% FBS. The dish should be approx 30% confluent for transfection.
2. Immediately before transfection, add chloroquine (25 μ*M* final concentration) to each plate (*see* **Note 3**).
3. Prepare the transfection mix by adding (per dish) 14 μg helper plasmid, 7 μg Env plasmid, 21 μg vector plasmid, and 389 μL 2 *M* $CaCl_2$ to a final volume of 3 mL H_2O (*see* **Note 10**).
4. Add an equal volume of 2X HBS to the transfection mixture while vortexing. Carefully add 6 mL of the transfection mixture to the cells.
5. After 12–18 h, aspirate media off dishes and add 30 mL of fresh DMEM/10% FBS per plate. When processing large numbers of plates, the amount of medium added can be reduced to 17 mL per plate in order to reduce the volume for the subsequent ultracentrifugation step.
6. Harvest the virus-containing media 48 h later. Centrifuge the virus-containing media at 500*g* for 10 min to remove cellular debris. Carefully decant the supernatant and filter it through a 0.45-μm filter.
7. Supernatant should be immediately concentrated or aliquoted, frozen on dry ice, and stored at –80°C.

3.3. Concentration of Lentiviral Supernatants by Ultracentrifugation

1. Transfer 0.45-μm-filtered lentiviral supernatant to a Beckman (Palo Alto, CA) polyallomer ultra-centrifuge tube (25 × 89 mm) and completely fill with DMEM/10% FBS medium (*see* **Note 11**). Centrifuge at 30,000*g* in a Beckman SW 28 rotor for 2 h at 15°C.
2. Carefully decant the supernatant and invert tubes on a paper towel for 1 min to remove most of the remaining liquid (*see* **Note 12**). A clear yellowish pellet should be visible.
3. Insert ultracentrifuge tube into a 50-mL conical tube with a cap and spin tube at 500*g* for 5 min at 15°C to pull down any remaining liquid.
4. Store tubes at 4°C for 2 h, vortexing every 15 min to resuspend the viral pellet (*see* **Note 13**).
5. Centrifuge tubes at 500*g* for 5 min to pull down all virus-containing liquid. Aliquot liquid into cryotubes and place cryotubes on dry ice until frozen (*see* **Note 14**). Virus is stored at –80°C.

3.4. Concentration of Lentiviral Supernatants by Ultrafiltration

1. Transfer up to 75 mL of 0.45-μm-filtered lentiviral supernatant to sample filter cup of a Centricon Plus-80 centrifugal filter device (*see* **Note 15**). Centrifuge at 3500*g* at 6°C in a swinging bucket rotor until all supernatant has passed into the filtrate collection cup (*see* **Note 16**).
2. Remove filter device from centrifuge and carefully separate sample filter cup from filtrate collection cup. Decant filtrate.
3. Insert retentate cup into the sample filter cup, invert, and centrifuge at 1000*g* for 2 min at 6°C.
4. Carefully remove sample filter cup from retentate cup and collect the concentrated sample.
5. Aliquot concentrated sample into cryotubes and place cryotubes on dry ice until frozen. Virus is stored at –80°C.

3.5. Concentration of Lentiviral Supernatants by Precipitation Using PEG

1. Transfer 0.45 μm filtered lentiviral supernatant to an appropriate size centrifuge container and add PEG 6000 (8.5% final concentration) and NaCl (0.4 *M* final concentration) to the supernatant and mix well by inverting (*see* **Note 17**).
2. Store at 4°C for 1.5 h, mixing every 20 to 30 min.
3. Centrifuge the container at 7000*g* for 10 min at 4°C. A white pellet should be visible.
4. Carefully decant the supernatant and resuspend the resulting pellet by vigorous pipetting in a small volume (200 μL per plate) of 50 m*M* Tris-HCl, pH 7.4. Vortex vigorously for 20 to 30 s to further resuspend the pellet (*see* **Note 18**).
5. Aliquot the resuspended virus pellet into cryotubes and place cryotubes in dry ice until frozen. Virus is stored at –80°C (*see* **Note 19**).

4. Notes

1. It is recommended that the production of HIV-1-based lentiviral vectors be carried out under conditions compatible with Biosafety Level 2 (BL-2) containments and practices.
2. This small-scale transfection protocol is used to quickly test and optimize components of the vector system.
3. There is no need for the medium to be changed prior to transfection. As it is thought that transfected DNA reaches the cell nucleus by transiting in the lysosomal compartment, chloroquine may inhibit possible DNA degradation by increasing the lysosomal pH.
4. Three-component HIV-1-based vector systems have been described ***(11,44,53)***. We use DNA that was purified using Qiagen kits. Alternative Env glycoproteins may require different concentrations of Env plasmid DNA. The optimal ratios need to be optimized on a case-by-case basis. Third-generation lentiviral vector systems that employ four-plasmid transfection protocols have been described and the ratios of the various plasmid DNAs were reported ***(12,13,54)***.
5. It is very important that the pH of the 2X HBS solution be adjusted precisely to 7.05 (within 0.05 pH units).
6. A fine precipitate should be visible under the microscope. Instead of DMEM/10% FBS, UltraCULTURE medium can be used at this point. We have found that 293T cells are not affected in their capacity to produce virus when kept in this medium. Sodium butyrate has been shown to increase the production of lentivirus-based vectors. Sodium butyrate treatment at an optimal concentration of 10 m*M* added 16 h after transfection for 8 h increased vector titers from 7- to over 20-fold ***(55)***.
7. Cells transfected with plasmids encoding VSV-G will not become confluent. VSV-G is highly fusogenic, and within 36 h after transfection, syncytia should appear in the transfected cell population. Most cells may eventually detach from the plate.
8. Frozen virus pseudotyped with VSV-G can be thawed and refrozen at least once without affecting titer ***(56)***. The stability of alternative lentiviral pseudotypes has not been determined.
9. VandenDriessche et al. ***(45)*** have used Nunc Cell Factories for lentivirus production at a 1 liter scale.
10. The 293 ALF10 cell line ***(44)*** is useful for the production of high-titer amphotropic (4070A) pseudotypes. Cells are plated 18 to 24 h prior to transfection. For transfection, 14 μg helper plasmid DNA and 21 μg of vector plasmid DNA are used per 150-mm dish.
11. Tubes should be completely filled as too little fluid in the tubes can result in their deformation at high speed.
12. One minute is usually sufficient. If the volume of the remaining liquid is too small, more DMEM/10% FBS should be added to effectively resuspend the pellet.

13. This should result in a homogeneous virus suspension. Prolonged incubation at 4°C was found not to be necessary. Farson et al. *(27)* have described an ultracentrifugation/resuspension procedure that involves two sequential ultracentrifugation steps. The final pellets were resuspended in PBS containing 1% FBS, shaken for 2 h at 4°C, and then flash frozen on dry ice and stored at –80°C.
14. Place tubes directly into crushed dry ice to freeze the virus as quickly as possible.
15. The ultrafiltration unit can be sterilized by rinsing it with 20 mL of 70% ethanol and spinning it at 3500*g* for 5 min at 15°C. The unit is then rinsed with PBS and spun again at 3500*g* for 5 min at 15°C. Centricon Plus-80 devices are compatible with both swinging-bucket and fixed-angle rotors. When using a fixed-angle rotor, rotate the device 180° halfway through the centrifugation to prevent clogging and to access the total membrane area.
16. This step may take up to 2 h. UltraCULTURE supernatants tend to run faster. Phenol red present in regular DMEM/10% FBS medium will concentrate along with the virus, causing the concentrated supernatant to appear dark red.
17. PEG 6000 and NaCl can be added to the lentiviral supernatant as solids or as solutions. If added as solids, stirring is necessary during the 1.5 h incubation period.
18. Small white flakes may be present after pipetting. Although vortexing allows further resuspension of the PEG pellet, prolonged vortexing may result in foaming that may inactivate the viral particles.
19. For in vivo applications, it may be desirable to perform a buffer exchange to remove the excess PEG and NaCl that may be present in the pellet.

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References

1. DuBridge, R. B., Tang, P., Hsia, H. C., Leong, P. M., Miller, J. H., and Calos, M. P. (1987) Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Mol. Cell Biol.* **7,** 379–387.
2. Soneoka, Y., Cannon, P. M., Ramsdale, E. E., et al. (1995) A transient three-plasmid expression system for the production of high titer retroviral vectors. *Nucleic Acids Res.* **23,** 628–633.
3. Gluzman, Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23,** 175–182.
4. Stratton, M. R., Reeves, B. R., and Cooper, C. S. (1989) Misidentified cell. *Nature* **337,** 311–312.
5. Page, K. A., Landau, N. R., and Littman, D. R. (1990) Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. *J. Virol.* **64,** 5270–5276.

6. Reiser, J., Harmison, G., Kluepfel-Stahl, S., Brady, R. O., Karlsson, S., and Schubert, M. (1996) Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proc. Natl. Acad. Sci. USA* **93,** 15,266–15,271.
7. Chang, L. J., Urlacher, V., Iwakuma, T., Cui, Y., and Zucali, J. (1999) Efficacy and safety analyses of a recombinant human immunodeficiency virus type 1 derived vector system. *Gene Ther.* **6,** 715–728.
8. Landau, N. R., Page, K. A., and Littman, D. R. (1991) Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. *J. Virol.* **65,** 162–169.
9. Akkina, R. K., Walton, R. M., Chen, M. L., Li, Q. X., Planelles, V., and Chen, I. S. (1996) High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *J. Virol.* **70,** 2581–2585.
10. Naldini, L., Blömer, U., Gallay, P., et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.
11. Mochizuki, H., Schwartz, J. P., Tanaka, K., Brady, R. O., and Reiser, J. (1998) High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells. *J. Virol.* **72,** 8873–8883.
12. Dull, T., Zufferey, R., Kelly, M., et al. (1998) A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* **72,** 8463–8471.
13. Hanawa, H., Kelly, P. F., Nathwani, A. C., et al. (2002) Comparison of various envelope proteins for their ability to pseudotype lentiviral vectors and transduce primitive hematopoietic cells from human blood. *Mol. Ther.* **5,** 242–251.
14. Carroll, R., Lin, J. T., Dacquel, E. J., Mosca, J. D., Burke, D. S., and St Louis, D. C. (1994) A human immunodeficiency virus type 1 (HIV-1)-based retroviral vector system utilizing stable HIV-1 packaging cell lines. *J. Virol.* **68,** 6047–6051.
15. Corbeau, P., Kraus, G., and Wong-Staal, F. (1996) Efficient gene transfer by a human immunodeficiency virus type 1 (HIV-1)-derived vector utilizing a stable HIV packaging cell line. *Proc. Natl. Acad. Sci. USA* **93,** 14,070–14,075.
16. Kaplan, A. H. and Swanstrom, R. (1991) The HIV-1 gag precursor is processed via two pathways: implications for cytotoxicity. *Biomed. Biochim. Acta* **50,** 647–653.
17. Rogel, M. E., Wu, L. I., and Emerman, M. (1995) The human immunodeficiency virus type 1 vpr gene prevents cell proliferation during chronic infection. *J. Virol.* **69,** 882–888.
18. Li, Y., Drone, C., Sat, E., and Ghosh, H. P. (1993) Mutational analysis of the vesicular stomatitis virus glycoprotein G for membrane fusion domains. *J. Virol.* **67,** 4070–4077.
19. Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
20. No, D., Yao, T. P., and Evans, R. M. (1996) Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* **93,** 3346–3351.
21. Yu, H., Rabson, A. B., Kaul, M., Ron, Y., and Dougherty, J. P. (1996) Inducible human immunodeficiency virus type 1 packaging cell lines. *J. Virol.* **70,** 4530–4537.

22. Kaul, M., Yu, H., Ron, Y., and Dougherty, J. P. (1998) Regulated lentiviral packaging cell line devoid of most viral cis- acting sequences. *Virology* **249,** 167–174.
23. Kafri, T., van Praag, H., Ouyang, L., Gage, F. H., and Verma, I. M. (1999) A packaging cell line for lentivirus vectors. *J. Virol.* **73,** 576–584.
24. Klages, N., Zufferey, R., and Trono, D. (2000) A stable system for the high-titer production of multiply attenuated lentiviral vectors. *Mol. Ther.* **2,** 170–176.
25. Xu, K., Ma, H., McCown, T. J., Verma, I. M., and Kafri, T. (2001) Generation of a stable cell line producing high-titer self-inactivating lentiviral vectors. *Mol. Ther.* **3,** 97–104.
26. Pacchia, A. L., Adelson, M. E., Kaul, M., Ron, Y., and Dougherty, J. P. (2001) An inducible packaging cell system for safe, efficient lentiviral vector production in the absence of HIV-1 accessory proteins. *Virology* **282,** 77–86.
27. Farson, D., Witt, R., McGuinness, R., et al. (2001) A new-generation stable inducible packaging cell line for lentiviral vectors. *Hum. Gene Ther.* **12,** 981–997.
28. Sparacio, S., Pfeiffer, T., Schaal, H., and Bosch, V. (2001) Generation of a flexible cell line with regulatable, high-level expression of HIV Gag/Pol particles capable of packaging HIV-derived vectors. *Mol. Ther.* **3,** 602–612.
29. Canivet, M., Hoffman, A. D., Hardy, D., Sernatinger, J., and Levy, J. A. (1990) Replication of HIV-1 in a wide variety of animal cells following phenotypic mixing with murine retroviruses. *Virology* **178,** 543–551.
30. Lusso, P., di Marzo Veronese, F., Ensoli, B., et al. (1990) Expanded HIV-1 cellular tropism by phenotypic mixing with murine endogenous retroviruses. *Science* **247,** 848–852.
31. Chesebro, B., Wehrly, K., and Maury, W. (1990) Differential expression in human and mouse cells of human immunodeficiency virus pseudotyped by murine retroviruses. *J. Virol.* **64,** 4553–4557.
32. Spector, D. H., Wade, E., Wright, D. A., et al. (1990) Human immunodeficiency virus pseudotypes with expanded cellular and species tropism. *J. Virol.* **64,** 2298–2308.
33. Zhu, Z. H., Chen, S. S., and Huang, A. S. (1990) Phenotypic mixing between human immunodeficiency virus and vesicular stomatitis virus or herpes simplex virus. *J. Acquir. Immune Defic. Syndr.* **3,** 215–219.
34. Landau, N. R. and Littman, D. R. (1992) Packaging system for rapid production of murine leukemia virus vectors with variable tropism. *J. Virol.* **66,** 5110–5113.
35. Sutton, R. E. and Littman, D. R. (1996) Broad host range of human T-cell leukemia virus type 1 demonstrated with an improved pseudotyping system. *J. Virol.* **70,** 7322–7326.
36. Stitz, J., Buchholz, C. J., Engelstadter, M., et al. (2000) Lentiviral vectors pseudotyped with envelope glycoproteins derived from gibbon ape leukemia virus and murine leukemia virus 10A1. *Virology* **273,** 16–20.
37. Christodoulopoulos, I. and Cannon, P. M. (2001) Sequences in the cytoplasmic tail of the gibbon ape leukemia virus envelope protein that prevent its incorporation into lentivirus vectors. *J. Virol.* **75,** 4129–4138.

38. Lewis, B. C., Chinnasamy, N., Morgan, R. A., and Varmus, H. E. (2001) Development of an avian leukosis-sarcoma virus subgroup A pseudotyped lentiviral vector. *J. Virol.* **75,** 9339–9344.
39. Beyer, W. R., Westphal, M., Ostertag, W., and von Laer, D. (2002) Oncoretrovirus and lentivirus vectors pseudotyped with lymphocytic choriomeningitis virus glycoprotein: generation, concentration, and broad host range. *J. Virol.* **76,** 1488–1495.
40. Kobinger, G. P., Weiner, D. J., Yu, Q. C., and Wilson, J. M. (2001) Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. *Nat. Biotechnol.* **19,** 225–230.
41. Chan, S. Y., Speck, R. F., Ma, M. C., and Goldsmith, M. A. (2000) Distinct mechanisms of entry by envelope glycoproteins of Marburg and Ebola (Zaire) viruses. *J. Virol.* **74,** 4933–4937.
42. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J. K. (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* **90,** 8033–8037.
43. Bartz, S. R. and Vodicka, M. A. (1997) Production of high-titer human immunodeficiency virus type 1 pseudotyped with vesicular stomatitis virus glycoprotein. *Methods* **12,** 337–342.
44. Reiser, J. (2000) Production and concentration of pseudotyped HIV-1-based gene transfer vectors. *Gene Ther.* **7,** 910–913.
45. VandenDriessche, T., Naldini, L., Collen, D., and Chuah, M. K. (2002) Oncoretroviral and lentiviral vector-mediated gene therapy. *Methods Enzymol.* **346,** 573–589.
46. Stein, C. S. and Davidson, B. L. (2002) Gene transfer to the brain using feline immunodeficiency virus-based lentivirus vectors. *Methods Enzymol.* **346,** 433–454.
47. Nomura, T., Yabe, T., Mochizuki, H., Reiser, J., Becerra, S. P., and Schwartz, J. P. (2001) Survival effects of pigment epithelium-derived factor expressed by a lentiviral vector in rat cerebellar granule cells. *Dev. Neurosci.* **23,** 145–152.
48. Cui, Y., Golob, J., Kelleher, E., Ye, Z., Pardoll, D., and Cheng, L. (2002) Targeting transgene expression to antigen-presenting cells derived from lentivirus-transduced engrafting human hematopoietic stem/progenitor cells. *Blood* **99,** 399–408.
49. Pham, L., Ye, H., Cosset, F. L., Russell, S. J., and Peng, K. W. (2001) Concentration of viral vectors by co-precipitation with calcium phosphate. *J. Gene Med.* **3,** 188–194.
50. Zhang, B., Xia, H. Q., Cleghorn, G., Gobe, G., West, M., and Wei, M. Q. (2001) A highly efficient and consistent method for harvesting large volumes of high-titre lentiviral vectors. *Gene Ther.* **8,** 1745–1751.
51. Scherr, M., Battmer, K., Blömer, U., et al. (2002) Lentiviral gene transfer into peripheral blood-derived CD34+ NOD/SCID-repopulating cells. *Blood* **99,** 709–712.
52. Cosset, F. L., Takeuchi, Y., Battini, J. L., Weiss, R. A., and Collins, M. K. (1995) High-titer packaging cells producing recombinant retroviruses resistant to human serum. *J. Virol.* **69,** 7430–7436.

53. Reiser, J., Lai, Z., Zhang, X. Y., and Brady, R. O. (2000) Development of multigene and regulated lentivirus vectors. *J. Virol.* **74,** 10,589–10,599.
54. Wu, X., Wakefield, J. K., Liu, H., Xiao, H., Kralovics, R., Prchal, J. T., and Kappes, J. C. (2000) Development of a novel trans-lentiviral vector that affords predictable safety. *Mol. Ther.* **2,** 47–55.
55. Sakoda, T., Kasahara, N., Hamamori, Y., and Kedes, L. (1999) A high-titer lentiviral production system mediates efficient transduction of differentiated cells including beating cardiac myocytes. *J. Mol. Cell Cardiol.* **31,** 2037–2047.
56. Higashikawa, F. and Chang, L. (2001) Kinetic analyses of stability of simple and complex retroviral vectors. *Virology* **280,** 124–131.

5

Detection and Titration of Lentivirus Vector Preparations

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1. Introduction

The accurate quantification of functional vector particles within a given vector preparation or stock is of fundamental importance to virtually all potential gene transfer applications for any viral vector of interest. This accuracy is especially critical if anticipated clinical applications will require scale-up of vector production or if multiple different vector stocks will be required for serial transductions of target cell populations over the course of a series of experiments. Standard notations designating the quantity of functional particles in a vector preparation include infectious units (i.u.) per milliliter and transducing units (TU) per milliliter ***(1)***. These units are interchangeable and represent the number of functional vector particles per volume of vector containing supernatant. The quantification of infectious or transducing units per unit volume allows transductions to be conducted based on the number of vector particles added per target cell and will therefore be standardized yielding more reproducible results. The numerical relationship between vector particles utilized per single target cell is designated as the multiplicity of infection (MOI). Establishing an appropriate MOI for a given transduction will, in part, depend on the desired transduction efficiency or, to an extent, the approximate number of chromosomal integrants desired per cell. Accurate vector stock titers will allow for the establishment of controlled transduction conditions and will, therefore, reduce experimental variability and simplify the interpretation of results obtained.

Consistent titer determinations are dependent on careful control of procedural variables throughout the titering process. Deviations from an established

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protocol will result in inconsistent titers and, subsequently, may have direct bearing on potential experimental designs. If meticulously adhered to, a given titering protocol will provide a reproducible, quantitative measure of the number of functional particles in a vector stock and should therefore allow for parallel experimentation with separate vector stocks and direct comparison of temporally separated experiments using the same or different vector stocks. Even when titering is conducted in a controlled manner, there will be slight deviation in calculated titers due to the inherent variability of the biological systems used to establish vector titer. It is, therefore, recommended to conduct multiple titer determinations with the same protocol so that several data points may be averaged to establish a more accurate titer for a single preparation of vector. It must also be noted that titering a vector stock using two different methodologies will likely result in two different titers. Again, this is due to the inherent variability in the nature of these assays. Regardless of the titering protocol used, the investigator should use the same titering protocol when characterizing different vector stocks to be utilized in serial or comparative experiments. As an important note, it is recommended that, if your laboratory receives a pretitered vector stock from another laboratory, the received vector should be retitered in house using a vector stock with a known titer as a control.

The methods by which most lentivirus-based vectors are titered are similar to established methods that have been used to titer retrovirus-based vectors for a number of years ***(1)***. Early on, most retrovirus-based vectors encoded a selectable resistance marker, such as neomycin phosphotransferase *(neo^r)*, that was used to determine the functional titer of a vector preparation ***(2–8)***. More recently, drug resistance genes, such as dihydrofolate reductase (L22Y variant) ($DHFR_{(L22Y)}$) ***(9–15)***, multidrug resistance 1 (MDR1) ***(16–18)***, and O^6-methylguanine DNA methyltransferase (MGMT and MGMT G156A variant) ***(19–21)***, have been incorporated into viral vectors allowing positive enrichment of transduced cells following drug resistance selection in vitro and in vivo. Similar to neomycin expressing vectors, vector that encode $DHFR_{(L22Y)}$, MDR1, or MGMT are titered based on growth of transduced cells in the presence of selective pressure. Of similar interest has been the incorporation of genes encoding cell surface markers and fluorescent or enzymatic proteins into lentivirus vectors. These latter modifications allow for the titration of vector stocks by flow cytometry or by direct fluorescence microscopy of transduced cells and provide very simple, convenient, and rapid ways in which titers can be obtained ***(22–28)***.

Recently, there has been increasing interest in the utilization of monocistronic vectors encoding only the gene of interest without a selectable marker or an indicator protein. In the absence of a marker that can be visualized or

detected in single cells or single cell-derived colonies, titers can be more difficult to determine and estimates of virus particles or of total viral protein in vector preparations have been used to normalize for virus ***(29)***. Alternatively, vector preparations have been normalized for the amount of viral RNA present by either slot blot or polymerase chain reaction (PCR) ***(30)***. Optimally, an approximate titer can be obtained by limiting dilution of the transduced cell population, followed by molecular analysis (PCR amplification), to determine directly the percentage of cells expressing the transgene of interest. Because these last determinations are very much gene specific and will vary from vector to vector and from gene to gene, these methods of titration are not usually standardized.

Because this chapter is intended to serve as a general guide, we only describe in detail the techniques employed in three methods of titration that can be used to determine the titer of a vector encoding any fluorescent cytoplasmic protein, cell surface protein, or a selectable marker. These methods may be readily adapted to determine titers of vectors encoding any cell fluorescent, cell surface, or resistance-conferring proteins. As general examples, we describe the methodology used to titer vectors that encode either green fluorescent protein (GFP), the human CD4 (hCD4) cell surface marker, or $DHFR_{(L22Y)}$.

2. Materials

1. Trypsin-ethylenediaminetetraacetic acid (EDTA) (GIBCO BRL, Carlsbad, CA).
2. Versene (EDTA) (0.2mg/mL) (BioWhitaker, Walkersville, MD).
3. Phosphate-buffered saline (PBS) (GIBCO BRL) without calcium chloride or magnesium chloride.
4. 4 mg/mL polybrene (Sigma, St. Louis, MO) in sterile water.
5. 5 m*M* azidothymidine (AZT) (Sigma) in PBS.
6. Levy hemacytometer (improved Neubauer) (Hausser Scientific, Morsham, PA).
7. 0.4% trypan blue stain (GIBCO BRL) dissolved in 1X PBS.
8. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (cDMEM).
9. HeLa cells (American Type Culture Collection [ATCC] No. CCL-2) grown in 100 × 20 mm tissue culture dishes (Beckton Dickinson, Franklin Lakes, NJ) in cDMEM at 37°C in a humidified incubator set at 5% CO_2.
10. Phycoerythrin (PE)-conjugated anti-hCD4 antibody (Beckton Dickinson).
11. Fluorescence-Activated Cell Sorter (FACS) buffer for flow cytometry samples (2% FBS in PBS).
12. 1% electron microscopy-grade paraformaldehyde solution in H_2O (PFA) (Electron Microscopy Sciences, Ft. Washington, PA) in PBS.
13. Thymidine phosphorylase (TP) (Sigma).
14. Neutrexin (trimetrexate glucuronate [TMTX] in H_2O) (US Bioscience, Cleveland, OH).
15. DHFR selection medium (DSM) (cDMEM supplemented with 10% TP-treated FBS, 1% P/S).

16. Coomassie blue stain (Sigma).
17. Six-well tissue culture plates (Beckton Dickinson).
18. 5-mL polystyrene round-bottom tubes (Beckton Dickinson).
19. Flow cytometry instrumentation and data analysis software. Flow cytometric data is acquired using a FACScan or FACSCalibur flow cytometer (Beckton Dickinson). Data analysis is performed using the CellQuest software package (Beckton Dickinson).

3. Methods

3.1. Titration of Vectors Encoding a Fluorescent Cytoplasmic Protein

3.1.1. Day 1

1. Plate 1×10^5 HeLa cells per well in 6-well plates using a total volume of 3 mL cDMEM per well and incubate overnight at 37°C with 5% CO_2.

3.1.2. Day 2

1. Aspirate media from one well and rinse the well by gently adding and aspirating 3 mL of PBS. Add 200 µL of trypsin-EDTA to dislodge the cells from the well and let trypsin-EDTA sit in the well for approx 2 min or until cells begin to release from the well surface. Gently aspirate trypsin, add 1 mL of cDMEM and pipet up and down until cells are in a single-cell suspension (*see* **Note 1**). Then place all resuspended cells (i.e., 1 mL) in a 5-mL polystyrene round-bottom tube.
2. Perform a viable cell count on the resuspended cells using a 1:10 dilution with 0.04% trypan blue stain (dilute stock 1:10 in PBS before mixing with cells) (*see* **Note 2**). Record this number for use as indicated in **Subheading 3.1.4., step 7**. Because cells from the count well were resuspended in 1 mL prior to counting, the cell count acquired is conveniently in cells per milliliter. Leave one well untransduced (mock) for unmarked cells to set the flow cytometer settings and to act as a control for the absence of vector (negative for reporter protein). This well does not receive vector (*see* **Note 2**).
3. Add 2 µL/mL of Polybrene (8 µg/mL) to all wells including mock for a final concentration of 4 µg/mL.
4. Add 10 µL/mL of AZT (5 m*M* stock, 50 µ*M* working concentration) to wells designated as controls and incubate for 30 min before adding vector (*see* **Note 3**).
5. Add concentrated vector stock (0.01 to 1 µL in 10-fold dilutions) to individual wells of HeLa cells in duplicate (*see* **Note 4**). If titering unconcentrated vector supernatant, add 10, 50, and 100 µL to individual wells of HeLa cells in duplicate. Add largest volume (1 mL) of vector input to cells pretreated with AZT.
6. Mix vector with medium by gently rocking each plate, avoiding circular motions so that the vector distributes evenly within the well.
7. Place plates of cells in a humidified incubator at 37°C with 5% CO_2 overnight.

3.1.3. Day 3

1. Aspirate medium from all wells and gently replace it with 3 mL of fresh cDMEM.

3.1.4. Day 5

1. Following the same procedure described on day two, remove the cells from each well with trypsin. Place the cells from each well into separate 5 mL polystyrene tubes, add 2 mL of FACS buffer and centrifuge for 400*g* for 5 min in a countertop centrifuge (*see* **Note 5**).
2. Carefully aspirate supernatant and resuspend cell pellets in 0.5 mL FACS buffer or 1% PFA (for sample storage for up to one week) and filter through a 100 μ*M* filter to remove clumps of cells (*see* **Note 6**).
3. Keep cells on ice or in a refrigerator until ready to analyze by flow cytometry.
4. Collect 5000 to 10,000 live events for each sample.
5. Analyze data obtained for all samples using a two-parameter dot plot diagram (*see* **Note 7**).
6. Using CellQuest, determine the percentage of EGFP positive events in each sample (*see* **Note 8**).
7. To calculate the titer (i.e., the number of infectious units per mL) of the vector stock, multiply the fraction of positive cells (5.0% = 0.05 EGFP+) in any given sample by the total number of cells on the infected plate (Day 2 cells) (as determined above on d 2) and divide this number by the amount of vector added in milliliters (volume).
   Titer = (%$EGFP^+$ cells/100)[Day 2 cell count (cells/mL)]/(volume of vector added [mL])
   Example: 5% $EGFP^+$ cells by flow cytometry following addition of 0.1 μL of vector to 1.7×10^5 Day 2 cells
   Titer = $[(5\%/100)(1.7 \times 10^5)]/(0.0001 \text{ mL}) = 8.5 \times 10^7$ i.u./mL
8. Average values determined for duplicate wells using the same volume of vector to establish titer (*see* **Note 9**).

3.2. Titration of Vectors Encoding a Cell Surface Protein

3.2.1. Days 1, 2, and 3

1. Conduct in the same manner as described in **Subheading 3.1.**

3.2.2. Day 5

1. Remove the cells from each well with approximately 200 μL of versene (EDTA) per well (*see* **Note 10**). Place the cells from each well into separate 5-mL polystyrene tubes, add 2 mL of FACS buffer, and centrifuge for 400*g* for 5 min in a countertop centrifuge.
2. Carefully aspirate supernatant and resuspend cell pellets in 0.2 mL FACS buffer.

3. Add the appropriate amount (according to the manufacturers instructions) of a fluorchrome-conjugated, antigen-specific antibody (anti-hCD4PE) to each tube including the mock transduced sample and incubate in the dark on ice for 30 min.
4. Add 2 mL of PBS supplemented with 1% FBS and centrifuge for 400*g* for 5 min in a countertop centrifuge.
5. Aspirate buffer and resuspend each cell pellet in PBS supplemented with 1% PFA. Then filter through a 100μ-filter to remove clumps of cells and keep cells in the dark and on ice or in a refrigerator until ready to analyze by flow cytometry.
6. Collect 5000 to 10,000 live events for each sample.
7. Analyze data obtained for all samples using a two-parameter dot plot (CellQuest) and determine the percentage of $hCD4^+$ events in each sample (*see* **Note 11**).
8. To calculate the titer (i.e., the number of infectious units per mL) of the vector stock, multiply the freaction of positive cells (5.0% = 0.05 $hCD4^+$) in any given sample by the total number of cells on the infected plate (Day 2 cells) (as determined above on d 2) and divide this number by the amount of vector added in milliliters (volume).
   Titer = {[%$hCD4^+$ cells/100][Day 2 cell count (cells/mL)]}/[volume of vector added (mL)]
   Example: 4% enhanced green fluorescent protein ($EGFP^+$) cells by flow cytometry following addition of 0.1 μL of vector to 1.8×10^5 Day 2 cells
   Titer = $[(4\%/100)(1.8 \times 10^5)]/(0.0001 \text{ mL}) = 7.2 \times 10^7$ i.u./mL
9. Average values determined for duplicate wells using the same volume of vector to establish titer.

3.3. Titration of Vectors Encoding $DHFR_{(L22Y)}$

3.3.1. Day 1

1. Plate HeLa cells as described in **Subheading 3.1., Day 1**. Cells should be plated and cultured in 3 mL DSM (*see* **Note 12**).

3.3.2. Day 2

1. Conduct transductions and cell count as described in **Subheading 3.1., Day 2**; however use lower amounts of concentrated vector (0.0001 and 0.001 μL) for transductions in duplicate.

3.3.3. Day 3

1. Remove the cells from each well with trypsin following the same procedure described on Day 2 in **Subheading 3.1.** Place the cells from each well into separate 5-mL polystyrene tubes, add 2 mL of PBS, and centrifuge for 400*g* for 5 min in a countertop centrifuge.
2. Resuspend cells in 3 mL DSM.

3. Plate cells from each transduction well at 1 : 10 and 1 : 50 dilution (300 and 60 µL) (duplicates for each dilution) in 3 mL total volume of DSM in a 6-well plate. Plate duplicate dilutions of mock transduced samples in a parallel plate.
4. Add TMTX to 1 µ*M* final concentration for both transduced and mock transduced samples (*see* **Note 13**).
5. Incubate cells for 11–14 d, checking medium daily. Change medium (DSM) if needed and supplement with the appropriate amount of TMTX. When no viable cells are visible in mock transduced wells, stain wells with coomassie blue stain in PBS, rinse wells with 3 mL PBS, and count colonies.
   Titer = (# colonies) (dilution factor)/([volume of vector added (mL)]
   Example: 10 colonies at 1 : 10 dilution following the addition of 0.001 µL of vector
   Titer = [(10)(10)]/(0.000001 mL) = 1×10^8 i.u./mL
6. Average values determined for duplicate wells using the same volume of vector to establish titer.

4. Notes

1. When observed under the microscope, cells will become detached from the plate and begin to take a round shape. It is very important that a single-cell suspension be obtained in order to facilitate the analysis and prevent the flow cytometer from clogging. These same steps will be repeated on d 5.
2. Even though 10^5 cells were originally plated, the day two cell number will have increased slightly, and therefore, in order to obtain an accurate titer, the number of Day 2 cells must be determined. This number must be documented because it will be used later to determine the actual number of infected cells following flow-cytometric analysis. When counting the cells using a hemacytometer, all 16 squares should be included, and the determination should be made using both chambers to obtain an average of these two numbers. In order to set up the flow cytometer for analysis, one well must be treated in the same manner as all others except for the addition of vector. This procedure will ensure that the appropriate settings are established in the flow cytometer so as to distinguish positive from negative cells. This distinction is particularly important when the mean fluorescence intensity levels are low.
3. Addition of AZT serves to block the process of reverse transcription, an essential step in the infectious process of all retroviruses, including all lentiviruses. Pretreatment of target cells with AZT will inhibit reverse transcription, and prevent transgene expression. This control allows the distinction between effective transduction and passive transfer of protein or DNA commonly referred to as pseudotransduction. Pseudotransduction can result in significant overestimation of vector titers. Addition of AZT prior to infection should result in approximately a 90% reduction in EGFP positive cells.
4. The need to titer different amounts of vector is to ensure that some of the determinations will fall within the linear range of the assay. For example, if

a vector stock has a titer of 108 i.u./mL, then 1 μL of this preparation should theoretically result in GFP expression in virtually all cells in that well. However, this is not the case, and as the number of green cells approaches 30 to 40%, it becomes unreliable for titer determination. Therefore lower concentrations of vector must be used to obtain an accurate titer (i.e., calculate titer using wells that contain between 1 and 10% green cells). Titers of concentrated vector stocks will vary significantly from vector to vector and from preparation to preparation. Once the same vector has been made one or two times, the number of dilutions tested can be reduced.

5. Centrifugation under these conditions should result in a relatively solid and visible pellet of cells at the bottom of the tube. However, care must be taken to avoid loss of cells in the pellet.
6. Care must be taken to introduce a single-cell suspension into the flow cytometer and to gate on and analyze only the live cell population. This distinction can usually be made by viewing a density plot as side scatter (SSC) versus forward scatter (FSC) for the control sample and gating on the population with intermediate SSC and FSC. This population will constitute 95% of the total data points displayed. Dead cells (very low SSC and FSC) should be omitted as they can have high levels of autofluorescence and may interfere with titer determinations.
7. The most common way to present single parameter flow cytometry data is a histogram plot of cell number vs relative fluorescence intensity. Setting the threshold of negative cells versus low expressing (but transduced) cells can be difficult if the majority of the cells express low levels of EGFP. In these cases, using a two-parameter dot plot facilitates the correct differentiation of negative vs positive cells.
8. Regardless of which type of plot is used, the percentage of transduced cells is determined by using the statistics function in the CellQuest software. When making this determination, care should be taken to consider only as positive those cells that are clearly brighter than the negative control. The percentage of cells in the negative control that are "fluorescent" should be less than 0.25%. This number should be subtracted from all determinations of percentage positive cells. This is particularly important for samples containing a low percentage of positive cells. These samples are likely to be in the linear range, but will also be the most affected by the background positive cells.
9. To obtain an accurate titer, calculations should be made using samples that are within the linear range of the assay (1 to 10% positive cells). This range becomes somewhat obvious after the initial determination of the percentage of positive cells in the different samples analyzed. Titers from two determinations using the same input vector are averaged. Subsequently, titers from two or more determinations made within the linear range of the assay, but obtained using different amounts of input vector, should be averaged. It is recommended to titer the same batch of vector at least two different times in this manner.
10. Versene (EDTA) is a nonproteolytic reagent for removing adherent cells from a solid surface. Use of trypsin to remove cells expressing a cell surface protein

from the transgene will result in its proteolytic degradation and the inability to distinguish transduced cells from untransduced cells. Due to the fact that versene is a more subtle method for removing adherent cells, gentle pipetting may be required in order to dislodge the cells and produce a single-cell suspension.

11. When calculating the percentage of antigen positive cells, care should be taken to consider only as positive those cells that are clearly brighter than the negative control (mock transduced cells) that has been stained with the fluorchrome-conjugated, target antigen-specific antibody. The percentage of cells in the negative control that are "fluorescent" should be less than 0.25%. This number should be subtracted from all determinations of percentage positive cells. This is particularly important for samples containing a low percentage of positive cells. These samples are likely to be in the linear range, but will likely be most affected by background "positive" cells.
12. Prepare DHFR selection medium by adding (1 U/mL of sterile filtered (0.1 μm) TP to 37°C FBS and incubating at 37°C for 2 h. In vitro, serum used in the culture medium can be depleted of thymidine nucleosides by TP treatment ***(9)***. This is critical for efficient selection because early progenitor cells resist antifolates such as TMTX by utilizing nucleoside salvage pathways to bypass the *de novo* synthesis block ***(10,31–33)***. When used in combination, TMTX and TP treatment prevents both *de novo* synthesis and salvage of thymidine and purine nucleosides. The resulting depletion of intracellular thymidine interrupts DNA maintenance and replication causing cell death. Depletion of serum thymidine nucleosides used in combination with TMTX therefore results in highly efficient selection against cells not expressing $DHFR_{(L22Y)}$.
13. TMTX is marketed under the brand name Neutrexin and comes as lyophilized powder (stored at –80°C), with each vial containing 25 mg. Resuspend lyophilized TMTX in 1.35 mL of sterile ddH_2O in the product vial. It is critical to avoid resuspending TMTX in solutions containing Cl- ions (PBS, etc.). Transfer solution to a 5-mL polystyrene tube and add 2 mL of ddH_2O to produce a 20 m*M* solution. Dilute this to 500 μ*M* stock solution (dilute 25 μL of 20 m*M* solution per mL ddH_2O) at –20°C. Add 2 μL/mL of the 500 μ*M* stock to DSM to achieve a final concentration of 1 μ*M*.

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References

1. Miller, A. D., Miller, D. G., Garcia, J. V., and Lynch, C. M. (1993) Use of retroviral vectors for gene transfer and expression. *Methods Enzymol.* **217,** 581–599.
2. Cone, R. D. and Mulligan, R. C. (1984) High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. *Proc. Natl. Acad. Sci. USA* **81,** 6349–6353.

3. Hock, R. A. and Miller, A. D. (1986) Retrovirus-mediated transfer and expression of drug resistance genes in human haematopoietic progenitor cells. *Nature* **320,** 275–277.
4. Miller, A. D., Eckner, R. J., Jolly, D. J., Friedmann, T., and Verma, I. M. (1984) Expression of a retrovirus encoding human HPRT in mice. *Science* **225,** 630–632.
5. Miller, A. D., Law, M. F., and Verma, I. M. (1985) Generation of helper-free amphotropic retroviruses that transduce a dominant-acting, methotrexate-resistant dihydrofolate reductase gene. *Mol. Cell Biol.* **5,** 431–437.
6. Miller, A. D., Ong, E. S., Rosenfeld, M. G., Verma, I. M., and Evans, R. M. (1984) Infectious and selectable retrovirus containing an inducible rat growth hormone minigene. *Science* **225,** 993–998.
7. Stuhlmann, H., Cone, R., Mulligan, R. C., and Jaenisch, R. (1984) Introduction of a selectable gene into different animal tissue by a retrovirus recombinant vector. *Proc. Natl. Acad. Sci. USA* **81,** 7151–7155.
8. Williams, D. A., Lemischka, I. R., Nathan, D. G., and Mulligan, R. C. (1984) Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse. *Nature* **310,** 476–480.
9. Allay, J. A., Persons, D. A., Galipeau, J., et al. (1998) In vivo selection of retrovirally transduced hematopoietic stem cells. *Nat. Med.* **4,** 1136–1143.
10. Allay, J. A., Spencer, H. T., Wilkinson, S. L., Belt, J. A., Blakley, R. L., and Sorrentino, B. P. (1997) Sensitization of hematopoietic stem and progenitor cells to trimetrexate using nucleoside transport inhibitors. *Blood* **90,** 3546–3554.
11. Corey, C. A., DeSilva, A. D., Holland, C. A., and Williams, D. A. (1990) Serial transplantation of methotrexate-resistant bone marrow: protection of murine recipients from drug toxicity by progeny of transduced stem cells. *Blood* **75,** 337–343.
12. Evans, J. T., Cravens, P., Gatlin, J., Kelly, P. F., Lipsky, P. E., and Garcia, J. V. (2001) Pre-clinical evaluation of an in vitro selection protocol for the enrichment of transduced CD34+ cell-derived human dendritic cells. *Gene Ther.* **8,** 1427–1435.
13. Gatlin, J., Douglas, J., Evans, J. T., Collins, R. H., Wendel, G. D., and Garcia, J. V. (2000) In vitro selection of lentivirus vector-transduced human CD34+ cells (In Process Citation). *Hum. Gene Ther.* **11,** 1949–1957.
14. Lewis, W. S., Cody, V., Galitsky, N., et al. (1995) Methotrexate-resistant variants of human dihydrofolate reductase with substitutions of leucine 22. Kinetics, crystallography, and potential as selectable markers. *J. Biol. Chem.* **270,** 5057–5064.
15. Spencer, H. T., Sleep, S. E., Rehg, J. E., Blakley, R. L., and Sorrentino, B. P. (1996) A gene transfer strategy for making bone marrow cells resistant to trimetrexate. *Blood* **87,** 2579–2587.
16. Blau, C. A., Neff, T., and Papayannopoulou, T. (1997) Cytokine prestimulation as a gene therapy strategy: implications for using the MDR1 gene as a dominant selectable marker. *Blood* **89,** 146–154.
17. Schiedlmeier, B., Kuhlcke, K., Eckert, H. G., Baum, C., Zeller, W. J., and Fruehauf, S. (2000) Quantitative assessment of retroviral transfer of the human

multidrug resistance 1 gene to human mobilized peripheral blood progenitor cells engrafted in nonobese diabetic/severe combined immunodeficient mice. *Blood* **95,** 1237–1248.

18. Sorrentino, B. P., Brandt, S. J., Bodine, D., et al. (1992) Selection of drug-resistant bone marrow cells in vivo after retroviral transfer of human MDR1. *Science* **257,** 99–103.
19. Allay, J. A., Davis, B. M., and Gerson, S. L. (1997) Human alkyltransferase-transduced murine myeloid progenitors are enriched in vivo by BCNU treatment of transplanted mice. *Exp. Hematol.* **25,** 1069–1076.
20. Davis, B. M., Reese, J. S., Koc, O. N., Lee, K., Schupp, J. E., and Gerson, S. L. (1997) Selection for G156A O6-methylguanine DNA methyltransferase gene-transduced hematopoietic progenitors and protection from lethality in mice treated with O6-benzylguanine and 1,3-bis(2-chloroethyl)-1- nitrosourea. *Cancer Res.* **57,** 5093–5099.
21. Maze, R., Kapur, R., Kelley, M. R., Hansen, W. K., Oh, S. Y., and Williams, D. A. (1997) Reversal of 1,3-bis(2-chloroethyl)-1-nitrosourea-induced severe immunodeficiency by transduction of murine long-lived hemopoietic progenitor cells using O6-methylguanine DNA methyltransferase complementary DNA. *J. Immunol.* **158,** 1006–1013.
22. Douglas, J., Kelly, P., Evans, J. T., and Garcia, J. V. (1999) Efficient transduction of human lymphocytes and CD34+ cells via human immunodeficiency virus-based gene transfer vectors. *Hum. Gene Ther.* **10,** 935–945.
23. Evans, J. T., Kelly, P. F., O'Neill, E., and Garcia, J. V. (1999) Human cord blood $CD34^{+}CD38^{-}$ cell transduction via lentivirus-based gene transfer vectors. *Hum. Gene Ther.* **10,** 1479–1489.
24. Gatlin, J., Melkus, M. W., Padgett, A., Kelly, P. F., and Garcia, J. V. (2001) Engraftment of NOD/SCID mice with human CD34(+) cells transduced by concentrated oncoretroviral vector particles pseudotyped with the feline endogenous retrovirus (RD114) envelope protein. *J. Virol.* **75,** 9995–9999.
25. Gatlin, J., Padgett, A., Melkus, M. W., Kelly, P. F., and Garcia, J. V. (2001). Long-term engraftment of nonobese diabetic/severe combined immunodeficient mice with human CD34+ cells transduced by a self-inactivating human immunodeficiency virus type 1 vector. *Hum. Gene Ther.* **12(9),** 1079–1089.
26. Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. USA* **93,** 11,382–11,388.
27. Naldini, L., Blomer, U., Gallay, P., et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.
28. Ranga, U., Woffendin, C., Yang, Z. Y., et al. (1997) Cell and viral regulatory elements enhance the expression and function of a human immunodeficiency virus inhibitory gene. *J. Virol.* **71,** 7020–7029.
29. Granelli-Piperno, A., Zhong, L., Haslett, P., Jacobson, J., and Steinman, R. M. (2000) Dendritic cells, infected with vesicular stomatitis virus-pseudotyped HIV-1,

present viral antigens to CD4+ and CD8+ T cells from HIV-1-infected individuals. *J. Immunol.* **165,** 6620–6626.
30. Li, X., Mukai, T., Young, D., Frankel, S., Law, P., and Wong-Staal, F. (1998) Transduction of CD34+ cells by a vesicular stomach virus protein G (VSV-G) pseudotyped HIV-1 vector. Stable gene expression in progeny cells, including dendritic cells. *J. Hum. Virol.* **1,** 346–352.
31. Belt, J. A., Marina, N. M., Phelps, D. A., and Crawford, C. R. (1993) Nucleoside transport in normal and neoplastic cells. *Adv. Enzyme Regul.* **33,** 235–252.
32. Lynch, T. P., Paran, J. H., and Paterson, A. R. (1981) Therapy of mouse leukemia L1210 with combinations of nebularine and nitrobenzylthioinosine 5'-monophosphate. *Cancer Res.* **41,** 560–565.
33. Rongen, G. A., Smits, P., Ver Donck, K., et al. (1995) Hemodynamic and neurohumoral effects of various grades of selective adenosine transport inhibition in humans. Implications for its future role in cardioprotection. *J. Clin. Invest.* **95,** 658–668.

6

Detection and Selection of Lentiviral Vector-Transduced Cells

Yan Cui and Lung-Ji Chang

1. Introduction

Lentiviruses are members of a subgroup of enveloped Retroviridae. Lentivirus-based gene delivery vectors have gained popularity in gene therapy field because of their notable potentials in delivering and integrating transgenes into both mitotic active and inactive cells in vitro and in vivo, a characteristic that overcomes many of the barriers of therapeutic gene therapy application. In the past few years, different lentiviral vector systems have been developed based on primate and nonprimate lentiviruses, including human immunodeficiency virus type 1 (HIV-1), simian immunodeficiency virus (SIV), equine infectious anemia virus (EIAV), and feline immunodeficiency virus (FIV) (*see* **refs. *1–4***). However, HIV-1-based lentiviral vector remains the best-studied and well-developed lentiviral vector system that has shown high transduction efficiencies targeting many human and experimental animal cell types, including stem cells (embryonic and adult) ***(5–10)***, terminally differentiated somatic cells (i.e., neurons) ***(7,11,12)***, muscles ***(7,13,14)***, skin ***(15)***, liver ***(13,16,17)***, islet ***(18,19)***, lung epithelium ***(20,21)***, retina ***(22,23)***, primary T lymphocytes ***(24,25)***, and fetal tissues ***(21,26)*** (**Table 1**).

Like all retroviruses, the lentivirus genome consists of two copies of plus-stranded RNA, which is converted to proviral DNA by reverse transcription after infection and is subsequently stably integrated into the host cell genome (reviewed in **ref. *4***). Currently, most of the lentiviral vector systems divide the viral genome into three separate constructs/components: a transducing vector carrying the transgene in a packageable viral genome, a helper construct providing essential structural proteins for viral packaging, and an envelope

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Table 1
Relative Efficiencies of Transduction of Various Cell Lines and Primary Cells with VSV-G Pseudotyped Lentiviral Vectors[a]

	Cell type[b]	Relative efficiency (+ to ++++[c])
Cell lines		
(human)		
HEK293	embryonic kidney cells	++++
TE671	rhabdomyosarcoma cells	++++
HeLa	cervical adenocarcinoma	++++
HepG2	hepatoma cells	++++
IB3-1	CF[d] bronchial epithelial cells	+++
K562	chronic myelogenous leukemia	++++
U937	myeloid leukemia cells	++++
A20	B cell lymphoma	+
EBV blasts	EBV transformed B lymphoma	+++
(mouse)		
Renca	renal cell carcinoma	++++
CT26	colon carcinoma	++++
NIH3T3	fibroblasts	+++
BaF3	pre-B lymphoma	+++
TF1	proerythroblastic leukemia cells	++++
DC2.4	immortalized immature DC	++/+++
P19	embryonic carcinoma	+++
Primary cells		
(human)		
HUVEC	umbilical vein endothelial cells	+++
$CD34^+$ cells	hematopoietic stem/progenitor cells	++/+++[e]
monocytes	myeloid cells	++
DC	immature dendritic cells	+++
T cells	lymphocytes	+++[f]
B cells	lymphocytes	+
(other species)		
ESC	mouse embryonic stem cells	+++
neuron	rat neuronal cells	+++
liver stem cells	rat oval cells	+++
liver	primary rat hepatocytes	++
RPE	rat retinal pigment epithelial cells	++++
rod	chicken eye rod cells	++
bone marrow	mouse BM mixed population	+++
HSC	mouse hematopoietic stem/progenitor cells	++

[a]The results of primary cell transduction are only for reference comparison; they are based on in vivo or ex vivo culture transduced using HIV-1 vectors carrying different reporter genes.

Therefore, the efficiency can vary markedly depending on the type of reporter gene and the infection condition.

[b]If not specified, the cell types are referring to human cells.

[c]Relative efficiency is defined as: ++++ = >50% cells transduced at MOI of 1 to 5; +++ = >50% cells transduced at MOI of 5 to 10; ++ = >30% cells transduced at MOI of 10–20; and + = 1–20% cells transduced at MOI > 20.

[d]Cystic fibrosis.

[e]Transduction efficiency of cytokine-stimulated $CD34^+$ stem cells (+++) is higher than that of unstimulated cells (++).

[f]Both naive and activated human T lymphocytes can be transduced relatively easily with lentiviral vectors; the transgene expression level in naïve T cells, however, is very low and barely above background level.

construct for pseudotyping viruses to broaden their tropism **(Fig. 1)**. Due to the pathogenic nature of HIV-1, tremendous efforts have been invested into further improvement of the lentiviral vector system ***(7,27–35)***. By minimizing the HIV-1 sequences in the transducing vector, by eliminating all of the accessory genes and unnecessary viral sequences in the helper construct, and by testing novel envelopes to broaden or to specify tissue tropism, significant improvements in lentiviral safety, increased transduction efficiency, and targeted delivery/expression of transgene can be accomplished. To improve safety, HIV-1 sequences in the transducing vector have been minimized to less than 600 bp (Cui, Gay, and Chang, unpublished). In addition, U3 and U5 sequences of the long terminal repeats (LTRs) of the lentiviral vector have been further modified to eliminate lentiviral basal promoter, and different versions of self-inactivating (SIN) vectors have been studied. Besides the safety improvement of SIN vectors, removal of viral basal promoters also makes targeted/controlled transgene expression possible. For improvement of transduction efficiency, enhancement of nuclear import and viral infectivity is achieved by incorporating the HIV-1 central DNA flap. Further improvement in targeted gene transfer is under progress by engineering tissue-specific envelopes and promoters.

To determine the efficiency of transgene delivery and future therapeutic effectiveness, many different reporter gene systems have been developed. These include use of drug resistant genes, intracellular enzymes, cell surface proteins, and fluorescent proteins ***(16,36,37)***. The transduced cells can be detected by immunohistochemical staining using specific antibodies or by polymerase chain reaction (PCR) with high sensitivity. The major advantage of fluorescent protein is that gene expression can be easily monitored in live cells through fluorescent microscope or flow cytometric fluorescence-activated cell sorter (FACS) analysis without the need of terminating the transduced cells. In addition, the green fluorescent protein (GFP)-expressing cells can be sorted to high purity and monitored in subsequent experiments. Although initially very poor in fluorescent intensity, GFP has been modified to optimize codon usage

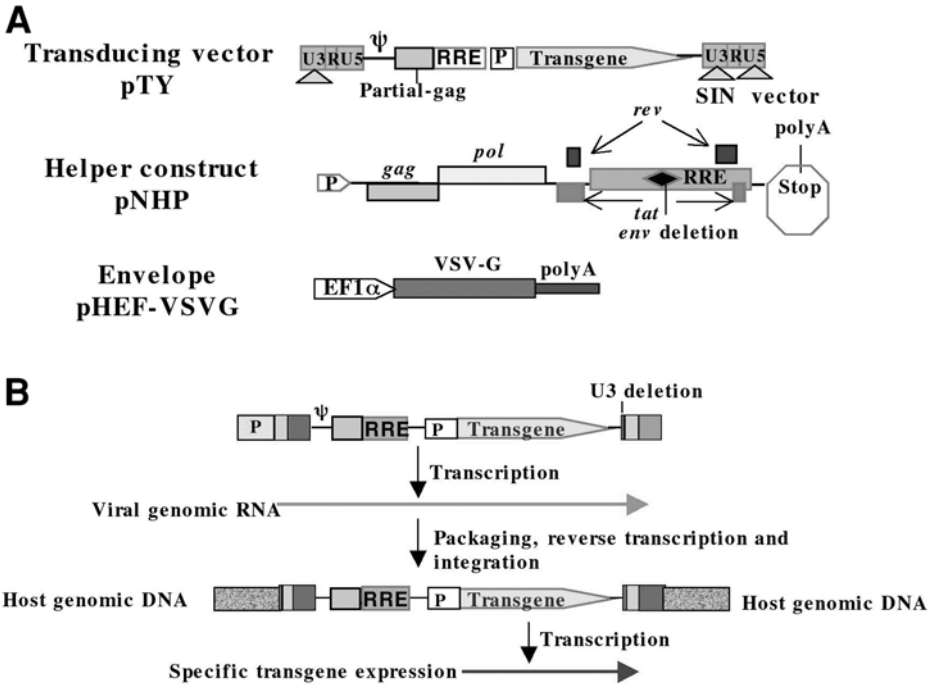

Fig. 1. Schematic illustration of HIV-1-based lentiviral vector system and transduction. **(A)** The HIV-1 vector system components. The basic vector system consists of three components: (1) transducing vector pTY, which contains modified 5′ long terminal repeats (LTR) and deletion of U3 in the 3′ LTR as self-inactivating (SIN) vector; packaging signal (Ψ); minimal HIV-1 *gag* sequence; an internal promoter (P) of non-HIV-1 origin; and gene of interest (transgene); (2) helper construct pNHP, which encodes HIV-1 Gag-Pol and regulatory proteins Tat and Rev, with all of the accessory genes deleted; (3) envelope construct pHEF-VSV-G, which encodes G protein of vesicular stomatitis virus to pseudotype viral particles for expanded tropism. **(B)** Illustration of lentiviral SIN vector infection/integration. SIN vector has its 3′ U3 deleted so that, after reverse transcription and integration, the proviral LTR promoter (copied from the 3′ U3) is disabled. Transgene is under control of the internal promoter in infected cells.

for human cells with significantly improved fluorescent intensity, enhanced GFP (EGFP), so that lentirival transduced cells having single copy of EGFP under the control of a strong promoter can be easily detected and monitored ***(7,38,39)***. Further engineering of fluorescent proteins that emit fluorescence signal at different wavelength has generated reporter genes that produce yellow fluorescent protein (YFP, Clontech), blue fluorescent protein (BFP, Clontech), cyan fluorescent protein (CFP, Clontech, Palo Alto, CA), and red fluorescent pro-

tein (DsRed, Clontech). Additionally, different colored fluorescent proteins as reporters have been tested in lentiviral vector system ***(37)***. These proteins are useful transgene monitoring tools for the study of various regulatory or biological conditions, and/or lineage diversity following lentiviral transduction.

Like fluorescent proteins, use of such cell surface proteins as truncated nerve growth factor receptor (NGFR) with only extracellular and transmembrane domain, influenza hemagglutinin (HA), mouse heat stable antigen (HSA) as reporters also allows easy identification and selection of the transgene-expressing cells without the need of fixing the cells or terminating experiments. The cells transduced with these reporter genes can be stained live with specific antibody conjugated with fluorochrome and then monitored and analyzed through flow cytometric analysis **(Fig. 2A)**. Alternatively, the transduced cells can be selected and enriched using immunomagnetic beads labeled with specific antibodies (e.g., Dynal, Biotech Inc., Lake Success, NY, or magnetic cell sorting [MACS] beads, Mittenyi Biotech Inc., Auburn, CA).

Use of reporter genes encoding enzymes, such as bacterial β-galactosidase (lacZ), placenta alkaline phosphatase (PLAP), and luciferase, is also popular for determining transgene efficiency. Analyses of expression of these reporter genes are based on the detection of a distinguishable colored product converted by the reporter enzyme from specific substrate. In addition, the sensitivity of the enzymatic assay can be increased by increasing the incubation time to enhance color development. Compared with the PCR method, which detects a single copy of the transgene at the DNA level or at the messenger RNA level by reverse transcription (RT)-PCR, both the enzymatic assay and the PCR method need to control for false positives that can be caused by endogenous enzyme activity and DNA contamination, respectively. The sensitivity of the enzymatic reporter gene system is higher when compared with the fluorescent protein and the antibody-staining methods. Like the multiple color fluorescent proteins, several enzymatic reporter genes can be combined and detected simultaneously using different colorimetric substrates (e.g., the co-expression of nuclear lacZ (nlacZ) and PLAP, **Fig. 2B**). However, most of these enzymatic reporter assays require the cells to be permeablized and/or fixed prior to the enzymatic reaction. Therefore, the transduction efficiency and other experimental readouts cannot be obtained unless the cells are terminated.

Each of the above systems has its own advantages and limitations and the choice of a reporter gene system is likely to be determined based on the requirements of an individual experiment and the background level of reporter gene in specific tissue type. With further improvement in the reporter gene technology, there is no doubt that more choices will be available combining the speed, convenience, viable sampling capacity and high sensitivity. In addition, live animal reporter systems, such as the positron emission tomography (PET)

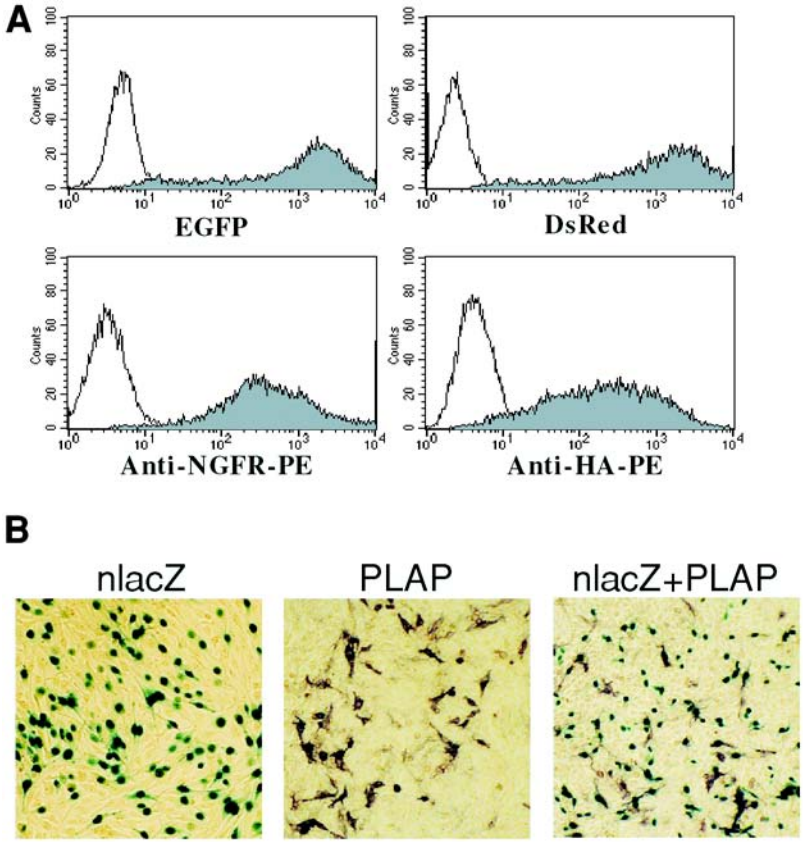

Fig. 2. Representative FACS analysis histograms and enzymatic assays of reporter genes in lentiviral transduced cells. Lentiviral vectors carrying **(A)** EGFP (upper left), DsRed (upper right), NGFR (lower left), and HA (lower right) and **(B)** nlacZ (left) and PLAP (middle) as reporter genes, and (right) cells coinfected with nlacZ and PLAP viral vectors. These reporter genes are under the control of an internal human elongation factor 1α promoter (EF1α) in human HEK293T (A) and TE671 (B) cells. Three days posttransduction, cells were harvested for FACS analysis of fluorescence protein expression (for EGFP and DsRed) or for primary and secondary antibody staining (for NGFR and HA) or stained for nlacZ and PLAP expression as described in Methods.

technology, are also gaining attention and can be very useful for in vivo transgene study ***(40–42)***.

Reporter genes or therapeutic genes with selective growth advantages represent another group of expression systems that have potential applications

for in vitro and in vivo studies of lentiviral gene transfer. When selection is desired, a drug-resistant gene, e.g., the one that confers resistance to G418 (the neomycin analog in the mammalian cells), hygromycin, bleomycin, or puromycin, can be incorporated into the vector, and only the transduced cell population will continue to grow in the presence of specific antibiotics. Other cases in which selective pressure may apply to nontransduced cells include knock-in to rescue a defective gene for the treatment of genetic diseases, such as the recent success in treating the Severe Combined Immunodeficiency-X1 (SCID-X1) disease patients with murine leukemia virus (MLV)-derived oncoretroviral vectors ***(43)***.

Besides direct physical separation, e.g., flow cytometric cell sorting, magnetic bead binding, and antibiotic resistance for the selection of transduced cells, the selection of target cells can also be based on specifically engineered targeting vectors through the use of different pseudotyped envelopes for specific cell attachment and entry. Alternatively, targeted gene expression can be controlled at transcriptional level using tissue specific promoters. Experimental results demonstrated that pesudotyped lentiviral vectors with various envelopes derived from amphotropic or ecotropic murine leukemia virus, vesicular stomatitis virus (VSV), gibbon ape leukemia virus (GALV), and feline endogenous virus RD114 may optimize lentiviral transduction in different subpopulations of cells ***(44,45)***. In addition, targeting transgene expression controlled at transcriptional level has been demonstrated using well-defined tissue-specific promoters ***(9,46,47)***. These approaches will have broad applications for treating diseases including stem cell and somatic cell genetic defects as well as cancers.

2. Materials

2.1. Cells and Cultures

1. Human embryonic kidney (HEK, 293, 293T, 293TF) cells.
2. Human rhabdomyosarcoma cells (TE671).
3. Human cervical carcinoma cells (HeLa).
4. Murine fibroblasts (NIH 3T3).

All these cell types can be obtained from American Type Culture Collection (ATCC), Rockville, MD. They are maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL) and 100 U/mL of penicillin-streptomycin (GIBCO BRL). SV40 T antigen expressing HEK 293 cells can be obtained from GenHunter (293T, Nashville, TN) or Invitrogen (293TF, Carlsbad, CA) and maintained in DMEM containing 10% FBS under G418 selection (400 μg/mL).

2.2. Plasmids

All plasmids used for transfection are purified by the CsCl gradient method *(48)* or using Qiagen (Valencia, CA) DNA Maxi-prep kits following the manufacture's instruction.

1. The lentiviral helper construct pHP encodeing HIV-1 Gag-Pol, essential for viral packaging *(7)*. pHP also encodes regulatory and accessory proteins, Tat, Rev, Vpr, Vif, and Vpu. The further improved helper construct pNHP encodes HIV-1 Gag-Pol, Tat, and Rev without the accessory genes and features additional deletion in the *env* gene *(49)*.
2. The pHEF-VSVG, encoding the vesicular stomatitis virus G protein (VSV-G) envelope.
3. The SIN lentiviral-transducing plasmid, pTY, carrying a reporter gene under a promoter of human elongation factor 1α gene (EF1α).
4. The pCEP4Tat, which can be included in DNA cotransfection to increase vector titer *(7)*.
5. The pTVEFeGFP, pTYEFeGFP, pTYEFnlacZ, pTYEF-PLAP, pTVEFHA, and pTVEFNGFR, lentiviral-transducing vectors carrying transgenes encoding EGFP, DsRed, nlacZ, PLAP, HA, or NGFR-extracellular and transmembrane anchorage domain.

All these lentiviral vectors, except for the transducing vectors carrying DsRed, PLAP, HA, or NGFR, can be obtained from the NIH AIDS Research and Reference Reagent Program (National Institutes of Health, Bethesda, MD).

2.3. DNA Transfection

1. Six-well tissue-culture plates.
2. Sterile 1.5-mL Eppendorf tubes (Brinkman, Westbury, NY) and 15-mL polystyrene or polycarbonate (not polypropylene) tubes.
3. Sterile double-distilled water (ddH_2O) (molecular biology grade) and serum-free DMEM.
4. Superfect transfection reagent (Quiagen).
5. Genejammer transfection reagent (Stratagene, La Jolla, CA).

2.4. Viral Vector Harvesting and Concentration

1. Sarstedt (Newton, NC) 1.5-mL sterile screw-cap tubes, 15- and 50-mL conical tubes.
2. Various sizes of 0.45-μm, sterile, low protein-binding filters (Millex-HV, Millipore, Boulder, CO).
3. Centricon P-20 plus (Millipore, MWCO 10,000).
4. Microfuge (max. 21,000*g*) and benchtop tissue culture centrifuge (Beckman, Fullerton, CA, or other venders).

2.5. Detection Assay for EGFP, DsRed, HA, and NGFR Reporter Genes

1. Six- or 12-well tissue culture plates.
2. Polybrene (Sigma), 8-mg/mL stock (1,000X), filter sterilized.
3. Falcon 12- × 75-mm polystyrene tubes.
4. Fluorescent-Activated Cell Sorter (FACS) buffer: 1X phosphate buffered saline (PBS) with 1% FBS, 0.1% NaN_3, and 2 m*M* ethylenediaminetetraacetic acid (EDTA) (optional).
5. Benchtop tissue culture centrifuge.
6. FACScan or FACSort (Becton Dickinson, San Jose, CA).
7. Biotinylated antireporter gene antibodies.
8. Streptavidin-conjugated phycoerythrin (PE).
9. FACS fixative solution: 1% formaldehyde in PBS.

2.6. Detection Assays for LacZ and PLAP Reporter Genes

1. Twenty-four-well tissue culture plates.
2. Polybrene (Sigma), 8 mg/mL stock (1000X), filter sterilized.
3. Sterile PBS.
4. Glutaraldehyde fixation solution: 1% formaldehyde (0.27 mL of 37.6% stock to make 10 mL), 0.2% glutaraldehyde (Sigma, 80 μL of 25% stock to make 10 mL) in PBS, freshly prepared.
5. X-Gal-staining solution: PBS containing 4 m*M* K-ferrocyanide (0.4 *M* stock in ddH_2O), 4 m*M* K-ferricyanide (0.4 *M* stock in ddH_2O), 2 m*M* $MgCl_2$ (1 *M* stock in ddH_2O), and 0.4 mg/mL 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) (20 mg/mL stock in dimethyl sulfide). Stock ferrocyanide, ferricyanide, and X-Gal should be kept frozen and away from light.
6. Alkaline phosphatase substrate kit: purchased from Vector Laboratories (Burlingame, CA) (BCIP/NBT, cat. no. SK-5400) containing levamisole and three reagent mixes.
7. Stock solution 0.1 *M* Tris-HCl (pH 9.5), autoclaved.
8. 37°C and 65°C incubators or water baths.

3. Methods

3.1. Generation of Recombinant Lentiviral Vectors

Viral vectors were generated by DNA cotransfection using four plasmids into 293, 293T, or TE671 cells. TE671 cells are easier to handle because they are more adherent to culture dishes than 293 cells. 293T cells, however, produce higher viral titer. We found that 293T cells after prolonged passage give the best transfection efficiency. All the following steps should be carried out in a biosafety hood and should follow NIH Biosafety Level 2 and 3 laboratory safety guidelines.

3.1.1. Transfection of TE671 Cells Using Genejammer (Stratagene)

1. The day (16–18 h) before transfection, seed 6-well plate with 7×10^5/well of TE671 cells in 1.5- to 2-mL of growth medium DMEM. By the time of transfection, the seeded cells should have reached approx 90–95% confluency (*see* **Note 1**).
2. Prepare helper DNA mix (at final concentration 1 μg/μL) as the following (per well): 1.8 μg pNHP, 0.3 μg pHEFVSV-G, 0.2 μg pCEP4tat, and 0.2 μg pHEFeGFP (*see* **Note 2**).
3. Mix 2.5 μL (2.5 μg) of the helper DNA and 1 μL (1 μg/μL) of the transducing vector pTY DNA in a sterile Eppendorf tube.
4. Into a 5-mL polystyrene tube, pipet 100 μL of serum-free DMEM and dispense 5 μL of GeneJammer solution to the center of the 100 μL DMEM, which is immediately mixed by gentle shaking.
5. Transfer the 3.5 μL of pHP/pTY DNA mix to the center of the DMEM-GeneJammer solution and shake the tube gently. The DNA-GeneJammer solution is kept at room temperature for 5 to 10 min.
6. Prior to transfection, the cells are fed with 900 μL per well of DMEM with 10% FBS.
7. The DNA-GeneJammer mix is added to each well dropwise with constant shaking and the plate is transferred a 3% CO_2 incubator at 37°C for 5 h (*see* **Note 3**).
8. Add an additional 1 mL of complete DMEM and continue incubation overnight.
9. The next morning, remove the culture media, wash the cells once with 1 mL medium, and change to fresh medium (1 to 2 mL) of choice. The transfection efficiency can be monitored under an inverted fluorescent microscope the next morning. The virus production peaks at 48 h after DNA-GeneJammer transfection. Virus production is markedly decreased after 60 h.

3.1.2. Transfection of 293T Cells Using Superfect

1. Split 293T cells (1×10^6 cells/well) into 6-well plates 17 to 18 h prior to Superfect DNA transfection.
2. Prior to transfection, the media is replaced with 600 μL/well of fresh complete DMEM with 10% FBS.
3. Add the following reagents to a sterile Eppendorf tube: 75 μL (per well) of serum-free DMEM, 2.7 μg of helper DNA mix (2.7 μL of the 1 μg/μL DNA mix: 1.8 μg pNHP, 0.5 μg pHEF-VSV-G, 0.2 μg pCEP4tat, and 0.2 μg pHEFeGFP as internal control) and 0.8 μg of pTY vector DNA; vortex briefly (*see* **Note 4**).
4. Add 7 μL per well of Superfect to the center of this DNA-DMEM solution (at a 2:1 ratio of Superfect to DNA) and mix immediately by pipetting up and down five times using a P1000 pipetman. The mixture sits at room temperature for 7–10 min.
5. Transfer approx 83 μL of DNA-Superfect mix dropwise to each culture well while swirling gently.

6. Incubate at 37°C in a 3% CO_2 incubator for 4 to 5 h.
7. Remove the complex-containing media, wash cells with desired growth medium, and feed the cells with 1.5 mL of the medium of choice at (e.g., AIM-V serum-free media for the human dendritic cell study) (*see* **Note 5**).
8. The culture is returned to a 3% CO_2 incubator overnight. Transfection efficiency can be monitored under an inverted fluorescent microscope the next morning. The virus production peaks between 24 and 36 h and declines rapidly after 48 h. The virus-containing supernatant can be harvested at 12 h intervals three times (up to 48 h) (*see* **Note 6**).

3.2. Lentivirus Harvesting and Concentration

3.2.1. Virus Harvesting

The virus supernatant is cleared of cell debris by centrifugation (1000*g*, 5 min) and filtered using a 0.45 μm low-protein binding filter. Virus should be stored in aliquots at –80°C. The transfected cells normally produce lentiviral vectors with titers ranging from 10^6 to 10^7 transducing units per milliliter of culture medium (*see* **Note 7**).

3.2.2. Lentiviral Vector Concentration by Microfuge Centrifugation

Small volumes of lentiviral vectors can be concentrated 30- to 50-fold by a simple centrifugation protocol using a microcentrifuge.

1. Filtered virus supernatant is transferred as 1-mL aliquot into a 1.5-mL sterile screw cap tube with the virus pelleting site marked.
2. With the marked side facing out, spin the tube at 20,000*g* at 4°C in a microfuge for 2.5 h.
3. Carefully transfer the tubes to a biosafety hood and discard most of the supernatant by pipeting, but leave behind approx 20 to 30 μL volume.
4. Rigorously vortex each tube for 20 s and vortex gently and continuously at 4°C in a mixer or shaker for 6 h or overnight (*see* **Note 8**).
5. Virus samples of the same preparation should be pooled and realiquoted to avoid titer variations among different tubes. The virus aliquots should be stored at –80°C.

3.2.3. Lentiviral Vector Concentration by Centricon Filtration

1. Wash the Centricon column with 70% ethanol and then sterile PBS twice in a biosafety hood.
2. Transfer 18 mL of virus supernatant to each Centricon P-20 plus column and centrifuge at 2500*g* for about 30 min or until the virus volume is reduced to 0.5 mL (*see* **Note 9**).
3. Flip the filter column and collect the concentrated virus onto the collection cup by centrifugation at 400*g* for 2 min and pool the concentrated virus.

3.3. Detection and Selection of Cells Transduced with Lentiviral Vectors Carrying HA, NGFR, GFP, DsRed, nlacZ, and PLAP Reporter Genes

3.3.1. Selection of Cells Transduced with Lentivirus Carrying a Reporter Gene Detectable by FACS

3.3.1.1. Lentiviral Transduction of Adherent Cells

1. Seed adherent cells, e.g., HeLa, 293T or NIH 3T3 cells, 1×10^5/well in 6-well plates. Assuming cell doubling time of 18–24 h, the actual cell number at the time of viral infection the second day will be 2×10^5/well (*see* **Note 10**).
2. On the second day, replace cell culture medium with 1.5 mL fresh medium containing 8 μg/mL polybrene. Add the viral inoculum to the cells and incubate at 37°C for 4 h.
3. At the end of the 4-h incubation, add at least one volume (1.5 mL) of fresh medium to dilute polybrene. The cells are cultured for 3–4 d before analysis or selection by FACS.

3.3.1.2. Lentiviral Transduction of Suspension Cells

1. On the day of transduction, prepare cells at 2×10^5/well in a 12-well plate or a Falcon 12- × 75-mm tube (#2054, for spin inoculation) for infection and incubate the cells at 37°C or centrifuge the cells at 150*g* at room temperature for 4 h (*see* **Note 11**).
2. At the end of the 4-h incubation/inoculation time, dilute polybrene by adding 1.5 mL fresh medium (as necessary) and culture the cells for additional 3–4 d.

3.3.1.3. Detection and Selection of Transduced Cells

1. Harvest lentiviral transduced adherent cells using trypsin or PBS containing 50 m*M* EDTA. Suspension cells are harvested by centrifugation in Falcon 12- × 75-mm tubes. Cells are washed twice with ice cold FACS buffer.
2. For cells transduced with HA or NGFR as reporter gene, resuspend the cells in 100 μL of cold FACS buffer, add appropriate amount of primary biotinylated-Ab (anti-HA or NGFR), and incubate on ice for 20 min. Wash the cells once with cold PBS, resuspend cells in 100 μL ice cold FACS, and add streptavidin-conjugated PE, or for immunomagnetic selection, add strepavidin-conjugated magnetic beads. Incubate on ice for 15 min and wash the cells twice.
3. Resuspend cells (Ab stained for HA or NGFR as reporter gene or directly harvested if EGFP and DsRed are used as reporter genes) in 0.5 to 1 mL of cold FACS fixative for FACS analysis. For viable selection of transduced cells, the labeled cells should not be fixed. Instead, the cells are resuspended in PBS and sorted with FACS sort or selected by magnetic affinity column.
4. GFP expression can be detected/selected with FL-1 channel and DsRed, HA, and NGFR with FL-2 channel of FACScan or FACSort **(Fig. 2)**.

3.3.2. Detection of Cells Transduced with Lentivirus Carrying Enzymatic Reporter Genes

3.3.2.1. lacZ Reporter Gene Assay

The method below detects cells transduced with lentiviral vectors carrying a nuclear lacZ reporter gene.

1. The transduced cells, in culture or in tissue section, may have strong endogenous lacZ activity that can result in a strong background staining, for examples, human primary hepatocytes, hematopoietic stem cells, and islets (beta cells). To inhibit endogenous lacZ activity, the enzymatic reaction should be performed under high pH. This can be easily achieved by adjusting the reaction to approx pH 8.5 using a Tris-HCl buffer.
2. The cells are fixed at room temperature with the glutaraldehyde fixative for exactly 5 min.
3. Wash cells three times with PBS and add 300 μL X-gal staining solution to the transduced cells and incubate at 37°C overnight.

3.3.2.2. PLAP Reporter Gene Assay

The PLAP reporter gene is even more sensitive then the lacZ reporter gene. For double staining, PLAP assay is performed after the lacZ reaction (skip **steps 1** and **2** below).

1. Transduced cells are washed with PBS 2 to 3 times.
2. Fix cells at room temperature with the formaldehyde fixative for 5 min.
3. Wash cells with PBS two to three times and heat the fixed cells in a 65°C incubator (or in a water bath with the plate tape sealed) under PBS for 30 min to inactivate endogenous alkaline phosphatase.
4. Prepare PLAP staining solution using the Alkaline Phosphatase Subtrate Kit IV (stored at 4°C). Briefly, to a 5 mL 100 m*M* Tris-HCl (pH 9.5), add a drop or less of Levamisole and mix well; add two drops of Reagent 1 and mix well; add two drops of Reagent 2 and mix well; add two drops of Reagent 3 and mix well.
5. Remove PBS from the cells, add 3 mL of PLAP staining solution and incubate at RT for 0.5–3 h away from light. If the reaction is weak, leave the reaction overnight at room temperature. If the reaction is strong, move the cells to 4°C overnight.

4. Notes

1. It is critical to transfect cells exactly 16–18 h after plating because the efficiency of transfection is critically dependent on the stage of cell cycle. In addition, mycoplasma contamination markedly reduces transfection efficiency; therefore, if necessary, cells should be treated with Normocin (InvivoGen, San Diego, CA) or mycoplasma removal reagent (ICN, Costa Mesa, CA).

2. The eGFP plasmid is included for monitoring transfection efficiency. If, however, the transducing vector already carries a fluorescent protein gene, the control pHEFeGFP plasmid should be omitted. The total DNA amount should remain the same, which can be adjusted using carrier DNA such as pUC19.
3. 3% CO_2 overnight during transfection increases transfection efficiency.
4. The DNA-transfection reagent mix can be proportionally scaled up. The 6-well plate protocol is preferred to larger flasks for the consistency of transfection.
5. AIM-V serum-free medium (Invitrogen, Carlsbad, CA) can be used in the 293T culture for the period of virus harvesting with no effect on cell adherence or viability. However, 293T cells will detach from the culture plates when they are maintained in AIM-V for extended periods of time.
6. The virus titer (yield) drops markedly after 60 h.
7. The virus supernatants should be kept on ice at all time; avoid frequent freezing and thawing since high temperature and frequent thawing affect virus titer ***(50)***.
8. Extensive vortexing is necessary for the full recovery of pelleted lentivirus.
9. The Centricon column can be reloaded more than once to concentrate more virus, although it takes a longer time to spin for the second round.
10. Different cell types are transduced by lentiviral vectors with different efficiencies. Therefore, whenever possible, it is preferential to determine the titer using the specific cell type of interest.
11. Spin inoculation may increase viral infectivity by 50–70%. The small-sized stem cells and lymphoma cell lines can survive the high speed spin (2000*g* for 4 h). On the other hand, dendritic cells, either primary or cell line, such as DC2.4 and activated human B lymphocytes, should be handled at low speed (150*g*).

References

1. Verma, I. M. and Somia, N. (1997) Gene therapy—promises, problems and prospects. *Nature* **389,** 239–242.
2. Amado, R. G. and Chen, I. S. (1999) Lentiviral vectors—the promise of gene therapy within reach? *Science* **285,** 674–676.
3. Federico, M. (1999) Lentiviruses as gene delivery vectors. *Curr. Opin. Biotechnol.* **10,** 448–453.
4. Chang, L.-J. and Gay, E. E. (2001) The molecular genetics of lentiviral vectors—current and future perspectives. *Curr. Gene Ther.* **1,** 237–251.
5. Uchida, N., Sutton, R. E., Friera, A. M., et al. (1998) HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **95,** 11,939–11,944.
6. Miyoshi, H., Smith, K. A., Mosier, D. E., Verma, I. M., and Torbett, B. E. (1999) Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* **283,** 682–686.
7. Chang, L.-J., Urlacher, V., Iwakuma, T., Cui, Y., and Zucali, J. (1999) Efficacy and safety analyses of a recombinant human immunodeficiency virus type 1 derived vector system. *Gene Ther.* **6,** 715–728.

8. Hamaguchi, I., Woods, N. B., Panagopoulos, I., et al. (2000) Lentivirus vector gene expression during ES cell-derived hematopoietic development in vitro. *J. Virol.* **74,** 10,778–10,784.
9. Lois, C., Hong, E. J., Pease, S., Brown, E. J., and Baltimore, D. (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295,** 868–872.
10. Wolfgang, M. J., Eisele, S. G., Browne, M. A., et al. (2001) Rhesus monkey placental transgene expression after lentiviral gene transfer into preimplantation embryos. *Proc. Natl. Acad. Sci. USA* **98,** 10,728–10,732.
11. Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. USA* **93,** 11,382–11,388.
12. Blomer, U., Naldini, L., Kafri, T., Trono, D., Verma, I. M., and Gage, F. H. (1997) Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J. Virol.* **71,** 6641–6649.
13. Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H., and Verma, I. M. (1997) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat. Genet.* **17,** 314–317.
14. Sakoda, T., Kasahara, N., Hamamori, Y., and Kedes, L. (1999) A high-titer lentiviral production system mediates efficient transduction of differentiated cells including beating cardiac myocytes. *J. Mol. Cell Cardiol.* **31,** 2037–2047.
15. Baek, S. C., Lin, Q., Robbins, P. B., Fan, H., and Khavari, P. A. (2001) Sustainable systemic delivery via a single injection of lentivirus into human skin tissue. *Hum. Gene Ther.* **12,** 1551–1558.
16. Pfeifer, A., Kessler, T., Yang, M., et al. (2001) Transduction of liver cells by lentiviral vectors: analysis in living animals by fluorescence imaging. *Mol. Ther.* **3,** 319–322.
17. Follenzi, A., Sabatino, G., Lombardo, A., Boccaccio, C., and Naldini, L. (2002) Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. *Hum. Gene Ther.* **13,** 243–260.
18. Gallichan, W. S., Kafri, T., Krahl, T., Verma, I. M., and Sarvetnick, N. (1998) Lentivirus-mediated transduction of islet grafts with interleukin 4 results in sustained gene expression and protection from insulitis. *Hum. Gene Ther.* **9,** 2717–2726.
19. Giannoukakis, N., Mi, Z., Gambotto, A., et al. (1999) Infection of intact human islets by a lentiviral vector. *Gene Ther.* **6,** 1545–1551.
20. Goldman, M. J., Lee, P.-S., Yang, J.-S., and Wilson, J. M. (1997) Lentiviral vectors for gene therapy of cystic fibrosis. *Hum. Gene Ther.* **8,** 2261–2268.
21. Tarantal, A. F., Lee, C. I., Ekert, J. E., et al. (2001) Lentiviral vector gene transfer into fetal rhesus monkeys (Macaca mulatta): lung-targeting approaches. *Mol. Ther.* **4,** 614–621.
22. Miyoshi, H., Takahashi, M., Gage, F. H., and Verma, I. M. (1997) Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. *Proc. Natl. Acad. Sci. USA* **94,** 10,319–10,323.

23. Bainbridge, J. W., Stephens, C., Parsley, K., et al. (2001) In vivo gene transfer to the mouse eye using an HIV-based lentiviral vector; efficient long-term transduction of corneal endothelium and retinal pigment epithelium. *Gene Ther.* **8,** 1665–1668.
24. Chinnasamy, D., Chinnasamy, N., Enriquez, M. J., Otsu, M., Morgan, R. A., and Candotti, F. (2000) Lentiviral-mediated gene transfer into human lymphocytes: role of HIV-1 accessory proteins. *Blood* **96,** 1309–1316.
25. Costello, E., Munoz, M., Buetti, E., Meylan, P. R., Diggelmann, H., and Thali, M. (2000) Gene transfer into stimulated and unstimulated T lymphocytes by HIV-1-derived lentiviral vectors. *Gene Ther.* **7,** 596–604.
26. Zahler, M. H., Irani, A., Malhi, H., et al. (2000) The application of a lentiviral vector for gene transfer in fetal human hepatocytes. *J. Gene Med.* **2,** 186–193.
27. Poznansky, M., Lever, A., Bergeron, L., Haseltine, W., and Sodroski, J. (1991) Gene transfer into human lymphocytes by a defective human immunodeficiency virus type 1 vector. *J. Virol.* **65,** 532–536.
28. Yu, H., Rabson, A. B., Kaul, M., Ron, Y., and Dougherty, J. P. (1996) Inducible human immunodeficienty virus type 1 packaging cell lines. *J. Virol.* **70,** 4530–4537.
29. Naldini, L., Blomer, U., Gallay, P., et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.
30. Dull, T., Zufferey, R., Kelly, M., et al. (1998) A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* **72,** 8463–8471.
31. Cui, Y., Iwakuma, T., and Chang, L. J. (1999) Contributions of viral splice sites and cis-regulatory elements to lentivirus vector function. *J. Virol.* **73,** 6171–6176.
32. Iwakuma, T., Cui, Y., and Chang, L. J. (1999) Self-inactivating lentiviral vectors with U3 and U5 modifications. *Virology* **261,** 120–132.
33. Lever, A. M. L. (2000) HIV RNA packaging and lentiviral-based vectors. *Adv. Pharmacol.* **48,** 1–28.
34. Kafri, T., van Praag, H., Gage, F. H., and Verma, I. M. (2000) Lentiviral vectors: regulated gene expression. *Mol. Ther.* **1,** 516–521.
35. Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., and Charneau, P. (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101,** 173–185.
36. Case, S. S., Price, M. A., Jordan, C. T., et al. (1999) Stable transduction of quiescent CD34(+)CD38(–) human hematopoietic cells by HIV-1-based lentiviral vectors. *Proc. Natl. Acad. Sci. USA* **96,** 2988–2993.
37. Hawley, T. S., Telford, W. G., and Hawley, R. G. (2001) "Rainbow" reporters for multispectral marking and lineage analysis of hematopoietic stem cells. *Stem Cells* **19,** 118–124.
38. Zolotukhin, S., Potter, M., Hauswirth, W. W., Guy, J., and Muzyczka, N. (1996) A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. *J. Virol.* **70,** 4646–4654.
39. Cui, Y., Golob, J., Kelleher, E., Ye, Z., Pardoll, D., and Cheng, L. (2002) Targeting transgene expression to antigen-presenting cells derived from lentivirus-transduced engrafting human hematopoietic stem/progenitor cells. *Blood* **99,** 399–408.

40. MacLaren, D. C., Gambhir, S. S., Satyamurthy, N., et al. (1999) Repetitive, non-invasive imaging of the dopamine D2 receptor as a reporter gene in living animals. *Gene Ther.* **6,** 785–791.
41. Sun, X., Annala, A. J., Yaghoubi, S. S., et al. (2001) Quantitative imaging of gene induction in living animals. *Gene Ther.* **8,** 1572–1579.
42. Gambhir, S. S., Barrio, J. R., Phelps, M. E., et al. (1999) Imaging adenoviral-directed reporter gene expression in living animals with positron emission tomography. *Proc. Natl. Acad. Sci. USA* **96,** 2333–2338.
43. Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., et al. (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288,** 669–672.
44. Hanawa, H., Kelly, P. F., Nathwani, A. C., et al. (2002) Comparison of various envelope proteins for their ability to pseudotype lentiviral vectors and transduce primitive hematopoietic cells from human blood. *Mol. Ther.* **5,** 242–251.
45. Stitz, J., Buchholz, C. J., Engelstadter, M., et al. (2000) Lentiviral vectors pseudotyped with envelope glycoproteins derived from gibbon ape leukemia virus and murine leukemia virus 10A1. *Virology* **273,** 16–20.
46. Yu, D., Chen, D., Chiu, C., Razmazma, B., Chow, Y. H., and Pang, S. (2001) Prostate-specific targeting using PSA promoter-based lentiviral vectors. *Cancer Gene Ther.* **8,** 628–635.
47. Lotti, F., Menguzzato, E., Rossi, C., et al. (2002) Transcriptional targeting of lentiviral vectors by long terminal repeat enhancer replacement. *J. Virol.* **76,** 3996–4007.
48. Sambrook, J. and Russell, D. W. (2001) Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
49. Zaiss, A.-K., Son, S., and Chang, L.-J. (2002) RNA 3′-readthrough of oncoretrovirus and lentivirus: implications in vector safety and efficacy. *J. Virol.* **76,** 7209–7219.
50. Higashikawa, F. and Chang, L.-J. (2001) Kinetic analyses of stability of simple and complex retroviral vectors. *Virology* **280,** 124–131.

7

Detection of Replication-Competent Lentiviral Particles

Harry Segall and Richard E. Sutton

1. Introduction

Lentiviral vectors derived from human immunodeficiency virus (HIV) have demonstrated exceptional promise as tools for gene therapy applications ***(1,2)***, but have also raised safety concerns because of potential creation of replication-competent lentivirus (RCL) by uncontrolled recombination ***(3–6)***. In order to be safe for clinical use, vector preparations must be formally and extensively tested to show they are absolutely free of RCL. Various methods have been proposed to detect RCL, all of which report high specificity and sensitivity and all of which can discriminate between replication-defective virus and RCL. There is currently a compelling need for standardization and validation of this method.

There are three previously described assays for RCL detection: (1) p24 (capsid) enzyme-linked immunosorbent assay (ELISA), (2) Tat-transactivation assay, and (3) marker-rescue assay ***(7–9)***. The first two only detect gene products of HIV and not actually RCL; the second assay is thought to be relatively insensitive, and none have been tested or validated. Analogous to work with murine leukemia virus vectors, the best method to detect RCL may be the marker-rescue assay ***(10,11)***, which is sensitive and discriminates replication-defective virus from RCL, generating a compelling need for its standardization and validation. Certain marker-rescue assay would also work for other lentiviral vectors (for which corresponding assays are still being developed), such as feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), and equine infectious anemia virus (EIAV), whereas the previously described assays for HIV RCL will work only with

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HIV-based vectors. Other investigators have used various assays for detection of RCL *(7–9)*. Their vector preparations are always found to be free of RCL, but they have never used a positive control to confirm that the assay was working or to assess its sensitivity.

The schematics of the marker-rescue assay are outlined below. A replication-defective HIV-based vector encoding human placental alkaline phosphatase (AP) was introduced by transduction into 293T cells, creating a stable marker-rescue cell line. Samples to be tested for the presence of RCL are used to transduce these 293T cells, and the cells are then passaged for a few weeks to allow washout of the initial supernatant and viral spread of any RCL. If RCL containing the necessary *trans* functions is present, it will act as a helper virus, providing proteins that will permit mobilization of the integrated provirus encoding AP. Supernatant from those 293T cells is next titered on naïve cells, which are subsequently stained for AP activity. Thus, the presence of RCL in the test sample will be consistent with the rescue of the AP virus from the 293T cells.

To test the sensitivity of this "AlkaRescue" assay, we created a replication-competent lentivirus, as a positive control, by introducing vesicular stomatitis virus G glycoprotein (VSV-G) into a first-generation HIV-based vector. This virus also carries enhanced yellow fluorescent protein (eYFP) for convenient monitoring of the viral spread. A variant of this virus, which has additional deletions of Vif and Vpr, was also used as a positive control. The negative control consisted of a VSV-G pseudotyped HIV-based vector, also carrying the eYFP marker. Characterization of these novel viruses will be reported separately.

2. Materials

2.1. Cell Lines

1. 293T and human osteosarcoma (HOS) cells are available through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Rockville, MD, www.aidsreagent.org). 293T cells are suitable for virus production by transient transfection.
2. 293T-AIB cells: 293T cells containing an integrated, replication-defective HIV encoding Gag, Pol, Tat, Rev, and a human placental alkaline phosphatase (AP)-internal ribosomal entry site (IRES)-blasticidin (AIB) resistance cassette; available from us. That combination of marker genes was chosen because (1) without selective pressure, AP expression was lost over a period of a few weeks, (2) it is highly unlikely those genes will be used in lentiviral clinical gene therapy trials, and iii) ease and sensitivity of AP staining of transduced cells.
3. U373-MAGI cells (from NIH AIDS Research and Reference Reagent Program). This is a sensitive indicator cell line for the HIV type 1 (HIV-1) Tat transactivator protein. The cells contain stably integrated, silent copies of the HIV-1 long

term repeat (LTR) promoter linked to the β-galactosidase gene. They produce β-galactosidase enzyme upon introduction of active Tat.

2.2. Maintenance of Cell Lines

1. HOS and 293T cells cultures are maintained in complete Dulbecco's modified Eagle's medium (DMEM) in a 5% CO_2 water-jacketed incubator kept at 37°C. HOS cells are adherent and require trypsinization for passage. They are quite hardy, fast-growing, and easily passaged at a 1:8 ratio twice weekly. 293T cells, which do not require trypsinization, should be passaged just prior to reaching confluence. Care should be taken so that they do not become overly confluent since that will reduce viability. For maximally healthy cells a split of 1:3 or 1:4 of a 70–80% confluent cell culture plate every 3–4 d should provide optimal cell growth conditions.
2. 293-AIB cells are maintained similarly to 293T cells. Passage every few months in Blasticidin (at 10 μg/mL) for 1 wk is recommended to enforce cassette gene expression. Cells can be stained periodically for AP expression, and all (>99%) should be positive.
3. U373-MAGI cells are maintained in complete DMEM and should be passaged twice weekly at a ratio of 1:10 just upon reaching confluency.

2.3. Reagents

1. DMEM (GIBCO, Gaithersburg, MD, or equivalent).
2. Fetal bovine serum (FBS) (GIBCO or equivalent) (heat inactivated, 56°C, 30 min).
3. Penicillin-Streptomycin (pen-strep) (GIBCO or equivalent): Concentration = 10,000 U Pen and 10 mg strep/mL.
4. Chloroquine (Sigma, St. Louis, MO), 1 m*M* prepared in distilled H_2O.
5. Blasticidins (Invitrogen, Costa Mesa, CA or ICN, Carlsbad, CA).
6. Polybrene (Sigma or equivalent).
7. Diethyl sulfoxide (DMSO) (Sigma or equivalent).
8. Trypsin-ethylenediaminetetraacetic acid (EDTA) solution: Trypsin 0.05%, EDTA 0.1% in phosphate-buffered saline (PBS).
9. Twelve-well flat bottom tissue-culture plates (Costar, Cambridge, MA).
10. 15-cm tissue-culture dishes (Costar).

2.4. Plasmids

Plasmids pHIV-VSV G-IRES-YFPΔEnv and pHIV-VSV G-IRES-eYFP ΔEnvΔVifΔVpr were constructed by inserting a VSV G-IRES-eYFP cassette in place of AP in the vectors pHIV-APΔEnv and pHIV-APΔEnv ΔVifΔVpr, respectively ***(12)***. The vector pHIV-IRES-eYFP was similarly constructed, based upon pHIV-APΔEnvΔVifΔVpr. For pHIV-AP IRES-blasti ΔenvΔVifΔVpr, the inserted cassette was AP-IRES-blasticidin resistance (the latter obtained from pcDNA6; Invitrogen). These are all available from us.

2.5. Additional Reagents for Calcium Phosphate Transfection

1. 2X HEPES-buffered saline (HBS): 16.4 g NaCl (0.28 *M* final), 11.9 g HEPES [N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (0.05 *M* final)], 0.21 g Na_2HPO_4 (1.5 m*M* final), 800 mL H_2O. Adjust pH 7.05 with 5 *N* NaOH. Accurate pH and the amount of phosphate are crucial. Add H_2O to 1 liter. Filter-sterilize and store at 4°C in aliquots.
2. 2.5 *M* $CaCl_2$: 183.7 g $CaCl_2$•$2H_2O$ (Sigma; tissue culture grade); H_2O to 500 mL. Filter-sterilize and store at 4°C in aliquots.
3. Sterile dH_2O.

2.6. Additional Reagents for Alkaline Phosphatase Staining

1. AP buffer: 1 mL of 1.0 *M* Tris-HCl pH 9.5 stock, 0.2 mL of 5.0 *M* NaCl stock, 0.1 mL of 1.0 *M* $MgCl_2$ stock, 0.1 mL of levamisole 24 mg/mL stock, dH_2O to 10 mL. Stable for about 6 mo at room temperature. Just before staining add fresh 44 µL/mL nitro blue tetrazolium (NBT) and 33 µL/mL 5-bromo, 4-chloro, 3-indolyphosphate (BCIP) (from NBT/BCIP combo, GIBCO, cat. no. 18280-016).
2. Fixative solution: 0.4% glutaraldehyde and 0.3% formaldehyde in PBS (for 500 mL PBS: 4.0 mL 50% glutaraldehyde, 4 mL of 37% formaldehyde, both available from many commercial suppliers).

2.7. Additional Reagents for X-gal Staining

1. Fix solution: 1% formaldehyde, 0.2% glutaraldehyde (in PBS). This solution can be made up in advance and stored at 4°C in the dark for approximately 1 mo.
2. Staining solution: to prepare 1.0 mL of staining solution, combine 950 µL PBS, 20 µL 0.2 *M* potassium ferrocyanide [$K_4Fe(CN)_6$], 20 µL 0.2 *M* potassium ferricyanide [$K_3Fe(CN)_6$], 1.0 µL 2.0 *M* Mg_2Cl, and 10 µL X-gal Stock. Store in dark at 4°C.
3. 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) stock: prepare at 40 mg/mL in DMSO. X-gal stock should be stored in the dark at –20°C. It will turn yellow over time, but this does not affect the assay. Discard the stock if it becomes greenish-brown.

2.8. Reagent Preparation

1. Complete medium: DMEM, 10% FBS, 1% Pen-strep, all sterile; store at 4°C.
2. Polybrene stock solution (5 mg/mL): dissolve 50 mg of polybrene into 10 mL PBS, filter-sterilize and store at 4°C. Aliquots are stable for 1 yr.

3. Methods

3.1. Preparation of Virus Stocks by Calcium Phosphate Transfection

A precipitate containing calcium phosphate and DNA is formed by slowly mixing a HEPES-buffered saline solution with a solution containing calcium chloride and DNA. This precipitate is directly layered onto the cells and adheres

to the surface of cells. It should be visible in the phase contrast microscope 30 min after transfection. Up to 50% of the cells on a dish will take up the DNA precipitate. Shocking the cells with DMSO may improve transfection efficiency (*see* **Note 1**).

1. The day before transfection, seed 15-cm tissue culture dishes with 3×10^6 293T cells in 15 mL complete DMEM. We have found that the highest transfection efficiencies are obtained with 293T cells that are 50–70% confluent at the time of transfection. After adding cells, gently shake the plates forward and backward, then side to side, three to four times. This process distributes cells evenly about the plate. Set plates in incubator and do not disturb overnight while they attach to plate.
2. Prepare the DNA for application to cells. Ethanol precipitate the DNA to sterilize it for transfection and then resuspend in sterile H_2O at a concentration of 4 μg/ml. Cotransfect cells with a mixture of 100 μg per plate of each plasmid DNA. To a 50-mL tube, add (per 15-cm plate, with all reagents at room temperature) 100 μg DNA (of each plasmid), 100 μL 2.5 *M* $CaCl_2$, dH_2O to 1 mL. To the DNA $CaCl_2$ mixture, add 1 mL 2X HBS dropwise while bubbling vigorously with automatic pipettor (keep eject button depressed).
3. Distribute the precipitate solution evenly onto cells and gently agitate to mix precipitate and medium. Observe the cells under a microscope; you should observe evenly distributed very small, black particles. Rock plates back and forth a few times to evenly distribute DNA/calcium phosphate particles; place back in 37°C incubator.
4. Add chloroquine to each plate to final concentration of 10 μ*M* (*see* **Note 2**). If one is making the RCL positive control, at this point the cells should be moved to a Biosafety Level (BL)3 laboratory.
5. Incubate the cells 5–8 h under standard growth conditions. Remove the medium and gently add 5 mL of 10% DMSO in PBS, allow to sit for 2 min, then aspirate, and replace with 40 mL complete DMEM.
6. Harvest the supernatant 3 d posttransfection and remove cell debris by centrifugation at 500*g* for 10 min.
7. Quantitate the number of transducing units per ml by titration on HOS cells. HOS cells are typically plated out the day before in 24-well format at 50,000 cells/well. Increasing amounts of vector supernatant are added (up to 0.1 mL) to each well and incubated overnight. The rest of the supernatant should be frozen at –80°C in appropriately sized aliquots. Cells are refed the following day and examined for transgene expression 48–96 h later. Titer is back-calculated to 1 mL. When RCL is being titered, any examination of cells should take place after fixation.

3.2. Detection of RCL

3.2.1. Viral Infection and Propagation of RCL

1. Prepare three 12-well plates. Label one plate as a positive control, one as a negative control, and one as the viral stocks to be tested.

2. Prepare test virus stock: the volume of test virus to be used for the infection depends on the degree to which the stock must be assayed; for research purposes 1–10% of the vector preparation should be used. Prepare a positive control stock that contains 10^3 to 10^4 IU of RCL in 1 mL. This stock is used to allow horizontal spread of the integrated AP-expressing provirus. Prepare eight 10-fold dilutions of this virus in 1 mL of complete DMEM in a 12-well plate to determine the sensitivity of the marker rescue assay. This is all performed in a B5L3 laboratory.
3. Prepare a negative control stock of VSV-G pseudotyped HIV-IRES-eYFP.
4. Prepare a suspension of 293-AIB cells in complete DMEM, at a density of 10^5 cells/mL. Add 1 mL of this cell suspension to each well containing viral stocks dilutions.
5. Place all three infected plates in a CO_2 incubator at 37°C. Passage the cells every 2–3 d at a split of 1:3 or 1:4. Higher cell density facilitates viral spread. During the assay spread of both helper and marker-containing viruses occurs.
6. At each passage, harvest 1 mL of cell supernatant from each well and store frozen at –80°C.
7. A reasonable time period for this experiment is 3–4 wk; in our experience if the test supernatant contains RCL, it will have spread by this time. Presence of RCL in the accumulated, stored supernatants may be determined by staining for AP (marker-rescue assay), by staining for β-galactosidase (Tat-transactivation assay) or by performing an ELISA for p24 (HIV capsid) protein, as described below.

3.2.2. AP Staining for Virus Titration (Marker-Rescue Assay)

1. Passage fresh, uninfected HOS cells 1:10 or 1:20 into 12-well plates the day before assaying the supernatants.
2. Use 1 mL of each supernatant to be tested to infect HOS cells and 72 h later carry out AP staining as described below.
3. Aspirate media from HOS cells and gently add 0.5 mL of fixative solution to each well. Incubate at room temperature for 10 min; then rinse with PBS.
4. Incubate plates at 65°C, for 20 min (to inactivate endogenous alkaline phosphatases).
5. Aspirate PBS and add 0.5 mL/well AP buffer. Incubate at room temperature for 30 min or longer (but typically no more than a few hours).
6. Score the number of positive cells (dark purple) in each well using an inverted light microscope.

3.2.3. X-gal Staining for Virus Titration (Tat-Transactivation Assay)

1. Plate MAGI indicator cells at 0.5×10^5 cells per well (24-well plate) or at 1×10^5 cells per well (12-well plate). The cells should be 30% confluent one day after plating.
2. One day after plating out the cells, remove the culture medium from the plated cells and add 0.5 mL to 1 mL of virus sample to each well.

3. Incubate the cells 48–72 h in a 37°C, 5% CO_2 incubator. The cells should be just subconfluent before staining.
4. Remove the culture medium and add 1–2 mL of fix solution to each well. Incubate for 5 min at room temperature (*see* **Note 3**).
5. Remove the fix and wash the cells three times with PBS (*see* **Note 4**). Add enough staining solution to each well to just cover the cells (about 250 μL/well for 12-well plate, 125 μL/well for 24-well plate). Incubate the cells at 37°C for 1 to 2 h, or longer, until the cells stain blue (*see* **Note 5**).
6. Check the cells under a light microscope for the development of blue color (positive cells will stain blue). Wash the plates twice with PBS. Count the number of blue-stained cells. Titer is expressed as the number of stained cells multiplied by the viral dilution.
7. Plates can be stored at +4°C in PBS with sodium azide or with 70% glycerol if a permanent record is desired. The color will not fade if the plates are kept from strong sunlight.

3.2.4. ELISA for p24 (Capsid) Protein Assay

For best sensitivity, we recommend the Coulter p24 ELISA kit (Beckman Coulter Inc, Brea, CA; cat. no. 6604535), used according to the detailed manufacturer's instructions.

4. Notes

1. Optimal transfection of 293T cells requires high-quality plasmid DNA, prepared by CsCl ultracentrifuge purification or Qiagen ion-exchange column chromatography (Qiagen Inc., Valencia, CA). Propagation of these plasmids is beyond the scope of this chapter, but initial samples may be obtained from us.
2. DNA delivered by calcium phosphate transfection is thought to transit through lysosomes to the nucleus, and chloroquine raises lysosomal pH, thus inhibiting lysosomal DNAses. Rigorous analysis suggests that chloroquine treatment (and the DMSO shock) is not strictly necessary, although refeeding the cells after 8 h is.
3. β-galactosidase activity decreases dramatically if the fix solution is left on for more than 10 min, presumably because the enzyme becomes inactivated.
4. Residual glutaraldehyde will interfere with color development.
5. Extending the incubation period may result in background staining. If cell confluency is high, do not stain cells for longer than 2 h before counting. This may result in a greater number of cells staining due to diffusion through gap-junctions. Cells may need to be stained overnight if the confluency or expression level is low. If your 37°C incubator is not humidified, seal the plate with parafilm to prevent it from drying out. X-gal staining may be performed using a commercial kit (Invitrogen, cat. no. K1465-01).

References

1. Buchschacher, G. L., Jr. and Wong-Staal, F. (2000) Development of lentiviral vectors for gene therapy for human diseases. *Blood* **95,** 2499–2504.

2. Trono, D. (2000) Lentiviral vectors: turning a deadly foe into a therapeutic agent. *Gene Ther.* **7,** 20–23.
3. Chong, H. and Vile, R. G. (1996) Replication-competent retrovirus produced by a 'split-function' third generation amphotropic packaging cell line. *Gene Ther.* **3,** 624–629.
4. Chong, H., Starkey, W., and Vile, R. G. (1998) A replication-competent retrovirus arising from a split-function packaging cell line was generated by recombination events between the vector, one of the packaging constructs, and endogenous retroviral sequences. *J. Virol.* **72,** 2663–2670.
5. Garrett, E., Miller, A. R., Goldman, J. M., Apperley, J. F., and Melo, J. V. (2000) Characterization of recombination events leading to the production of an ecotropic replication-competent retrovirus in a GP+envAM12-derived producer cell line. *Virology* **266,** 170–179.
6. Otto, E., Jones-Trower, A., Vanin, E. F., et al. (1994) Characterization of a replication-competent retrovirus resulting from recombination of packaging and vector sequences. *Hum. Gene Ther.* **5,** 567–575.
7. Kafri, T., van Praag, H., Ouyang, L., Gage, F. H., and Verma, I. M. (1999) A packaging cell line for lentivirus vectors. *J. Virol.* **73,** 576–584.
8. Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H., and Verma, I. M. (1998) Development of a self-inactivating lentivirus vector. *J. Virol.* **72,** 8150–8157.
9. Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. USA* **93,** 11,382–11,388.
10. Forestell, S. P., Dando, J. S., Bohnlein, E., and Rigg, R. J. (1996) Improved detection of replication-competent retrovirus. *J. Virol. Methods* **60,** 171–178.
11. Printz, M., Reynolds, J., Mento, S. J., Jolly, D., Kowal, K., and Sajjadi, N. (1995) Recombinant retroviral vector interferes with the detection of amphotropic replication competent retrovirus in standard culture assays. *Gene Ther.* **2,** 143–150.
12. Sutton, R. E., Wu, H. T., Rigg, R., Bohnlein, E., and Brown, P. O. (1998) Human immunodeficiency virus type 1 vectors efficiently transduce human hematopoietic stem cells. *J. Virol.* **72,** 5781–5788.

8

Lymphocytes

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1. Introduction

The manipulation of gene expression in primary lymphocytes has been shown to have potential therapeutic applications for immunodeficiencies ***(1–3)***, and cancer immunotherapy ***(4,5)***. Efficient gene transfer is an absolute prerequisite for developing successful clinical gene therapy protocols. At present, viral gene transfer vectors based on murine leukemia virus (MLV) are used in most clinical trials and have been used successfully to transduce cells from various tissues, including cells of hematopoietic origin ***(6–8)***. However, transduction of primary lymphocytes has been limited primarily by the requirement that the cells be proliferating for stable retroviral integration ***(9)***. Although potent induction of cell proliferation in vitro has been used to overcome this problem ***(1,10–12)***, it has now become evident that activation stimuli such as antibodies and mitogens result in alteration of the CD4/CD8 ratios and T-cell receptor (TCR) repertoire composition of T lymphocytes and induce changes of their cytokine secretion profile, thus precluding preservation of the pool of naïve lymphocytes and their functional integrity ***(13,14)***.

1.1. Lentiviral Vectors

The applicability and potential clinical benefits of gene therapy based on genetically modified T lymphocytes largely depend on gene transfer efficiency and on the overall quality of the reinfused T cells. Therefore, it would be desirable to avoid inducing cell division during the gene transfer procedures by minimal ex vivo manipulation. The recent development of lentiviral vectors based on human immunodeficiency virus type 1 (HIV-1) may provide a way to overcome this problem by taking advantage of the lentivirus characteristic

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mitosis-independent nuclear import of the preintegration complex and of its capability of infecting nonproliferating cells ***(15,16)***. Unlike other retroviruses, HIV-1 efficiently targets the nucleus of infected cells and encodes a viral integrase able to insert a provirus copy into the genome of nondividing cells ***(17–19)***. Wild-type HIV-1, however, has a limited host range and is generally produced at relatively low titers ***(20,21)***. These characteristics, in addition to its high pathogenicity are formidable obstacles to its direct application as a vector system. These problems have been circumvented by the generation of lentiviral vectors retaining the advantageous elements of HIV-1, but eliminating the viral determinants of pathogenesis including the HIV-1 envelope that is replaced with the surface vesicular somatitis virus G glycoprotein (VSV-G) ***(15,22)***.

Most commonly, lentiviral vectors are produced via a transient expression system using a three-plasmid cotransfection protocol ***(15)*** that consists of the following genetic elements: gene transfer construct, packaging construct, and envelope expression construct. The transfer construct contains, in addition to the gene of interest, the expression of which is driven by an internal promoter, *cis*-acting lentiviral sequences required for packaging, reverse transcription, and integration. The packaging construct encodes viral structural proteins and regulatory proteins, but lacks the viral envelope gene, the packaging signal, and the other viral sequences critical for reverse transcription and integration. Proteins expressed by the packaging construct genes (i.e., Gag/Pol, Tat, Rev ± Vpr, Vpu, Vif, Nef) have several functions among which the formation of the capsid structure and the recognition of specific cis-acting sequences in the lentiviral genomic RNA encoded by the transfer vector. This recognition results in reverse transcription, efficient transcription, and reliable cytoplasmic export of full-length vector transcripts. The envelope construct typically contains a heterologous envelope (e.g., VSV-G) that broadens the tropism of the vector. VSV-G-pseudotyped virions present a practical advantage in that they have a broad host range and stable physical properties that allow purification and concentration of high-titer vector preparations by ultracentrifugation ***(15,16,23)***. The different expression constructs are maintained in the form of bacterial plasmids and can be transfected into mammalian cells to produce replication-defective virus stocks.

1.2. Factors Controlling Lentiviral-Mediated Transduction of Primary Human Lymphocytes

For potential clinical applications, it is necessary that the desired genes are delivered effectively to the target cells in a safe and nontoxic manner. Because they are based on a highly pathogenic human virus, lentiviral vectors raise higher safety concerns than do standard retroviral vectors. Pathogenicity of

lentiviruses is largely attributed to the action of their components encoded by the accessory genes, Vpr, Vpu, Vif, and Nef ***(24,25)***. For this reason, several groups tested the possibility that accessory genes may be eliminated from lentiviral vector packaging constructs. These experiments demonstrated that multiple attenuated replication-defective lentiviral vectors devoid of all accessory genes could be produced without compromising viral titers (2×10^6 transducing units (TU)/mL) ***(22)*** while maintaining the capability of transducing such resting cell types as retinal cells, pancreatic islets, central nervous system neurons, and progenitor and differentiated hematopoietic cells ***(26–28)***. However, important gene transfer restrictions still apply to some nonproliferative tissues or cell types and recent studies have indicated that highly purified nonactivated primary human peripheral blood lymphocytes or monocytes were refractory to transduction by HIV-1-derived vectors.

Several lines of evidence suggest that lymphocytes may require host cellular factors and/or viral-associated mechanisms to relieve the block to efficient and stable gene delivery ***(29,30)***. Exogenous interventions, such as the addition of minimal combination of cytokines into the culture media during transduction, have been utilized to facilitate such mechanisms ***(31,32)***. Alternatively, gene transfer into noncycling T lymphocytes can be attempted in the presence of antigen-presenting cells (APCs) and including the HIV-1 accessory gene(s) in the virus-packaging plasmid used with the aim of generating cellular activation in response to the presentation of viral antigens ***(33–35)***. In addition to the above strategies, improved transduction methods, such as colocalization of virus and target cells on recombinant fibronectin and low speed centrifugation ("spinoculation"), have resulted in increased transduction efficiency of unstimulated peripheral blood lymphocytes (PBLs) ***(36–38)***. At such minimal ex vivo manipulation conditions, lentiviral vectors allow gene transfer in noncycling lymphocytes at levels markedly superior to those usually reached with MLV-derived vectors ***(32–35)***.

The use of markers that allow easy and quick identification of transduced cells (e.g., enhanced green fluorescent protein [EGFP], CD2, truncated nerve growth factor receptor [tNGFR]) have markedly improved our ability and precision to monitor the efficiency of in vitro gene transfer ***(10,39,40)***. A simple flow cytometry analysis allows quick comparison of various transduction protocols and the dissection of the relative transduction levels achieved in distinct T-cell subsets. In the following paragraphs of this chapter, we describe optimized conditions for efficient transduction and transgene expression in mitogen-stimulated, as well as unstimulated, primary human lymphocytes, using lentiviral vector encoding EGFP under the transcriptional control of the human phosphoglycerate kinase (PGK) promoter.

2. Materials

2.1. Plasmids

1. Lentiviral vector plasmid pHR′CMV-LacZ ***(15)*** is used as gene transfer vector with the following modifications. The cytomegalovirus (CMV) early promoter is replaced with the human PGK promoter. The 3.1kb β-galactosidase (LacZ) cDNA is replaced with the cDNA of EGFP (Clonetech, Palo Alto, CA).
2. The packaging plasmids pCMVΔR8.2 ***(15)*** (carrying all the HIV-1 accessory genes, Vif, Vpu, Vpr, and Nef) and pCMVΔR8.91 ***(22)*** (devoid of all HIV-1 accessory genes) are used to express the HIV-1 *gag*, *pol*, *tat*, and *rev* genes and thereby produce lentiviral structural and regulatory proteins.
3. The plasmid pMD.G ***(15)***, carrying the VSV-G coding sequence driven by the CMV promoter and followed by the β-globin polyadenylation site, is used to pseudotype vector particles.

2.2. Culture Media and Reagents

1. RPMI 1640 (Life Technologies, Gaithersburg, MD).
2. Dulbecco Modified Eagle's Medium (DMEM) (Life Technologies).
3. L-glutamine (Life Technologies).
4. Fetal bovine serum (FBS) (Gemini-Bioproducts, Calabasas, CA).
5. Ficoll-Paque (Organon Teknika, Durham, NC).
6. Phosphate-buffered saline (PBS) (Life Technologies).
7. Trypsin (0.25%, Life Technologies).
8. Trypan blue, dissolved at 0.1% in 1X PBS.
9. Propidium iodide (Sigma, St. Louis, MO), dissolved at 30 μg/mL in 1X PBS.
10. Ethanol 80%.
11. Phytohemagglutinin (PHA, Sigma), dissolved in sterile bi-distilled water at 1 mg/mL, filtered 0.22 μm, aliquoted, and stored at –20°C (Sigma).
12. Recombinant human interleukin (rhIL)-2 (PeproTech, Norwood, MA), dissolved in sterile 1X PBS at 10^5 U/mL, aliquoted, and stored at –20°C.
13. Poly-L-Lysine-coated plates (10-cm diameter, Biocoat, Beckton Dickinson, Bedford, MA).
14. p24 gag enzyme-linked immunosorbent assay (ELISA) kit (Coulter Diagnostics, Hialeah, FL).
15. ABOTT LAB Polybrene.
16. RNase A (Sigma).
17. 0.45-μm filters (Nalgene, Rochester, NY).
18. 300-mm diameter plastic dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ).
19. 100-mm diameter plastic dishes (Falcon, Becton Dickinson).
20. Human fibronectin coated 6-well cell culture plates (Biocoat, Becton Dickinson, San Jose, CA).
21. Fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson, San Jose, CA).
22. 2% paraformaldehyde (Sigma) in 1X PBS.

23. FACSCalibur and CellQuest software (Becton Dickinson, San Jose, CA) for immunophenotypic characterization and analysis.
24. 293T cells are grown in DMEM, supplemented with 10% FBS and 2 mM L-glutamine (Life Technologies), and can be obtained from American Type Culture Collection (ATCC, Rockville, MD).
25. Fresh peripheral blood (PB) samples, obtained from healthy, HIV-seronegative donors.

3. Methods

3.1. Purification of Adherent Cell-Depleted Lymphocytes

1. Recover mononuclear cells (MNCs) from blood samples by Ficoll gradient centrifugation. Briefly, heparinized peripheral blood is diluted 1:1 with RPMI 1640 and overlayed onto Ficoll-Paque solution in 15-mL tubes. After 30 min of centrifugation at 500*g*, MNCs are recovered from the interface and washed twice in PBS.
2. Resuspend MNCs obtained by Ficoll separation in RPMI 1640 containing 1% heat-inactivated FBS and incubate on 30-mm diameter plastic dishes at 37°C for 3 h. Then, collect the nonadherent cells and incubate once again at 37°C for 12 to 20 h to eliminate completely adherent cells. Purity of the isolated lymphocytes is usually greater than 98% as determined by immunophenotyping and flow cytometry.
3. To obtain mitogen-activated lymphocytes, cultivate PBLs in the presence of 5 μg/mL of PHA and 100 IU/mL of rhIL-2 for 3 d. Thereafter, cell cultures should be maintained in RPMI 1640 containing 10% FBS and 100 IU/mL of rhIL-2.
4. To assess the purity and activation status of the target lymphocyte population, stain 5×10^5 adherent cells-depleted, resting, or mitogen-stimulated lymphocytes with fluorescein isothiocyanate (FITC)- and/or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs, Becton Dickinson, San Jose, CA) for the lineage specific human markers CD3, CD4, CD8, CD19, CD13, CD14, and CD56 and the cell activation markers CD69, CD25, and CD71. After transduction, cells are stained with the same mAbs on d 5 posttransduction.
5. After staining and washing, fix the cells in 4% paraformaldehyde and acquire 5000 to 10,000 events with a FACSCalibur and analyze using the CellQuest software. Isotype FITC- and/or PE-conjugated control antibodies are used to assess specificity of immunostaining procedures and to set marker regions.

3.2. Transduction of PBLs

Methods for producing and titrating lentivirus vector preparations are described in previous chapters of this book.

Transductions of lymphoid cells (*see* **Notes 1–3**) are performed on fibronectin-coated 6-well plates in the presence of 5 μg/mL polybrene at a multiplicity of infection (MOI) of approx 10–50 (*see* **Notes 4** and **5**) as determined on proliferating 293T cells.

1. Infect cells with the viral supernatant at a density of 10^6 cells/well in 3 mL final volume.
2. Centrifugate at 800*g* for 30 min at 32°C ("spinoculation").
3. Afterward, allow transductions to proceed for additional 48 h at 37°C, 5% CO_2.
4. After transduction, wash cells three times with PBS, trypsinize for 5 min to remove potential unadsorbed viral particles, and reincubate in fresh culture medium. In the case of resting lymphocytes, after transduction cells are cultured either in the absence of stimulation for 5–10 d or stimulated with PHA (5 μg/mL) and rhIL-2 (100 U/mL) for 3 d, culture in the presence of rhIL-2 (100 U/mL) and stimulate with PHA every 2 wk.
5. Harvest periodically transduced cells for the analysis of marker gene (e.g., flow cytometry analysis for EGFP expression) (*see* **Notes 6** and **7**).

3.3. Cell Cycle Analysis

Cell cycle status can be monitored by measuring cellular DNA content using propidium iodide (PI) staining and flow cytometry analysis ***(41)***.

1. Wash cells obtained pre- and posttransduction in PBS three times and resuspend in ice-cold 80% ethanol.
2. Treat cells with 1 mL of PBS containing 180 U of RNase A at 37°C for 30 min.
3. Stain cells with PBS containing 30 μg/mL of PI.
4. Analyze the cellular DNA content by using a FACSCalibur. Data acquisition and analysis are performed with the CellQuest and ModFit *LT* softwares, respectively.

4. Notes

1. Handling of lentiviral vectors is classified at P2 containment and should always be carried out in laminar flow hoods. Human blood samples are to be considered biohazard material and should be handled with universal precaution under Biosafety Level 2.
2. All solid waste should be autoclaved and liquid waste treated with 10% bleach. To avoid cross-contamination, it is advisable to segregate lentiviral work from routine cell culture using separate laminar flow hoods and incubators.
3. Although mitogen-activated lymphocytes could be transduced efficiently with viral stocks produced either in the presence or absence of HIV-1 accessory genes in the packaging construct, our experience indicates that, for efficient transduction of nonactivated primary lymphocytes, it is always necessary to use the viral stocks generated in the presence of all the viral accessory genes.
4. Repeat freeze and thaw of viral stocks results in decrease of virus titer. It is, therefore, recommended to prepare small aliquots of 100–500 μL/tube. Thawing of viral stocks is best performed at 37°C.
5. Since lentiviral particles are generally pseudotyped with the VSV-G envelope, transduction independently from the presence of a specific receptor occurs on the target cells, an MOI of 10–50 is sufficient to transduce 50–80% of lymphocytes.

If the MOI is too high (>100), lentiviral vector transduction may result in cytopathic effects on lymphocytes.

6. Early after exposure to lentiviral vectors, gene expression can be observed even in the absence of stable integration (pseudotransduction). To reduce the problem of overestimation of transduction efficiency due to pseudotransduction, transduced cells should be cultured at least for 48–72 h prior to analysis of transgene expression. At this time point, gene expression due to pseudotransduction will be significantly lower.
7. The procedures described herein results in transduction rates greater than 50% in both $CD4^+$ and $CD8^+$ subsets of resting and mitogen-activated lymphocytes as determined by FACS analysis. To achieve these results, however, it is important to use high-titer viral stocks ($>10^6$ TU/mL).

References

1. Blaese, R. M., Culver, K. W., Miller, A. D., et al. (1995) T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science* **270,** 475–480.
2. Woffendin, C., Ranga, U., Yang, Z., Xu, L., and Nabel, G. J. (1996) Expression of a protective gene prolongs survival of T cells in human immunodeficiency virus-infected patients. *Proc. Natl. Acad. Sci. USA* **93,** 2889–2894.
3. Chinen, J., Aguilar-Cordova, E., Ng-Tang, D., Lewis, D. E., and Belmont, J. W. (1997) Protection of primary human T cells from HIV infection by Trev: a transdominant fusion gene. *Hum. Gene Ther.* **8,** 861–868.
4. Hwu, P., Shafer, G. E., Treisman, J., et al. (1993) Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor gamma chain. *J. Exp. Med.* **178,** 361–366.
5. Hwu, P. and Rosenberg, S. A. (1994) The genetic modification of T cells for cancer therapy: an overview of laboratory and clinical trials. *Cancer Detect. Prev.* **18,** 43–50.
6. Bordignon, C., Notarangelo, L. D., Nobili, N., et al. (1995) Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients. *Science* **270,** 470–475.
7. Kohn, D. B., Weinberg, K. I., Nolta, J. A., et al. (1995) Engraftment of gene-modified umbilical cord blood cells in neonates with adenosine deaminase deficiency. *Nat. Med.* **1,** 1017–1023.
8. Dunbar, C. E., Cottler-Fox, M., O'Shaughnessy, J. A., et al. (1995) Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. *Blood* **85,** 3048–3057.
9. Miller, D. G., Adam, M. A., and Miller, A. D. (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol.* **10,** 4239–4242.
10. Mavilio, F., Ferrari, G., Rossini, S., et al. (1994) Peripheral blood lymphocytes as target cells of retroviral vector-mediated gene transfer. *Blood* **83,** 1988–1997.
11. Quinn, E. R., Lum, L. G., and Trevor, K. T. (1998) T cell activation modulates retrovirus-mediated gene expression. *Hum. Gene Ther.* **9,** 1457–1467.

12. Pollok, K. E., van der Loo, J. C., Cooper, R. J., Kennedy, L., and Williams, D. A. (1999) Costimulation of transduced T lymphocytes via T cell receptor-CD3 complex and CD28 leads to increased transcription of integrated retrovirus. *Hum. Gene Ther.* **10,** 2221–2236.
13. Seder, R. A. (1994) Acquisition of lymphokine-producing phenotype by CD4+ T cells. *J. Allergy Clin. Immunol.* **94,** 1195–1202.
14. Dietrich, P. Y., Walker, P. R., Schnuriger, V., et al. (1997) TCR analysis reveals significant repertoire selection during in vitro lymphocyte culture. *Int. Immunol.* **9,** 1073–1083.
15. Naldini, L., Blomer, U., Gallay, P., et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.
16. Reiser, J., Harmison, G., Kluepfel-Stahl, S., Brady, R. O., Karlsson, S., and Schubert, M. (1996) Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proc. Natl. Acad. Sci. USA* **93,** 15,266–15,271.
17. Gallay, P., Swingler, S., Aiken, C., and Trono, D. (1995) HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. *Cell* **80,** 379–388.
18. Gallay, P., Swingler, S., Song, J., Bushman, F., and Trono, D. (1995) HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell* **83,** 569–576.
19. Bukrinsky, M. I. and Haffar, O. K. (1997) HIV-1 nuclear import: in search of a leader. *Front. Biosci.* **2,** d578–d587.
20. Page, K. A., Landau, N. R., and Littman, D. R. (1990) Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. *J. Virol.* **64,** 5270–5276.
21. Freed, E. O., Delwart, E. L., Buchschacher, G. L. Jr., and Panganiban, A. T. (1992) A mutation in the human immunodeficiency virus type 1 transmembrane glycoprotein gp41 dominantly interferes with fusion and infectivity. *Proc. Natl. Acad. Sci. USA* **89,** 70–74.
22. Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L., and Trono, D. (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat. Biotechnol.* **15,** 871–875.
23. Akkina, R. K., Walton, R. M., Chen, M. L., Li, Q. X., Planelles, V., and Chen, I. S. (1996) High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *J. Virol.* **70,** 2581–2585.
24. Cullen, B. R. (1998) HIV-1 auxiliary proteins: making connections in a dying cell. *Cell* **93,** 685–692.
25. Emerman, M. and Malim, M. H. (1998) HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. *Science* **280,** 1880–1884.
26. Miyoshi, H., Takahashi, M., Gage, F. H., and Verma, I. M. (1997) Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. *Proc. Natl. Acad. Sci. USA* **94,** 10,319–10,323.

27. Uchida, N., Sutton, R. E., Friera, A. M., et al. (1998) HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **95,** 11,939–11,944.
28. Mochizuki, H., Schwartz, J. P., Tanaka, K., Brady, R. O., and Reiser, J. (1998) High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells. *J. Virol.* **72,** 8873–8883.
29. Fauci, A. S., Pantaleo, G., Stanley, S., and Weissman, D. (1996) Immunopathogenic mechanisms of HIV infection. *Ann. Intern. Med.* **124,** 654–663.
30. Kinoshita, S., Chen, B. K., Kaneshima, H., and Nolan, G. P. (1998) Host control of HIV-1 parasitism in T cells by the nuclear factor of activated T cells. *Cell* **95,** 595–604.
31. Chun, T. W., Engel, D., Mizell, S. B., Ehler, L. A., and Fauci, A. S. (1998) Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines. *J. Exp. Med.* **188,** 83–91.
32. Unutmaz, D., KewalRamani, V. N., Marmon, S., and Littman, D. R. (1999) Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. *J. Exp. Med.* **189,** 1735–1746.
33. Kitchen, S. G., Korin, Y. D., Roth, M. D., Landay, A., and Zack, J. A. (1998) Costimulation of naive CD8(+) lymphocytes induces CD4 expression and allows human immunodeficiency virus type 1 infection. *J. Virol.* **72,** 9054–9060.
34. Zhang, Z., Schuler, T., Zupancic, M., et al. (1999) Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science* **286,** 1353–1357.
35. Chinnasamy, D., Chinnasamy, N., Enriquez, M. J., Otsu, M., Morgan, R. A., and Candotti, F. (2000) Lentiviral-mediated gene transfer into human lymphocytes: role of HIV-1 accessory proteins. *Blood* **96,** 1309–1316.
36. Hanenberg, H., Xiao, X. L., Dilloo, D., Hashino, K., Kato, I., and Williams, D. A. (1996) Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat. Med.* **2,** 876–882.
37. Pollok, K. E., Hanenberg, H., Noblitt, T. W., et al. (1998) High-efficiency gene transfer into normal and adenosine deaminase- deficient T lymphocytes is mediated by transduction on recombinant fibronectin fragments. *J. Virol.* **72,** 4882–4892.
38. Fehse, B., Schade, U. M., Li, Z., et al. (1998) Highly-efficient gene transfer with retroviral vectors into human T lymphocytes on fibronectin. *Br. J. Haematol.* **102,** 566–574.
39. Dardalhon, V., Noraz, N., Pollok, K., et al. (1999) Green fluorescent protein as a selectable marker of fibronectin-facilitated retroviral gene transfer in primary human T lymphocytes. *Hum. Gene Ther.* **10,** 5–14.
40. Cheng, L., Fu, J., Tsukamoto, A., and Hawley, R. G. (1996) Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells. *Nat. Biotechnol.* **14,** 606–609.
41. Telford, W. G., King, L. E., and Fraker, P. J. (1992) Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry. *Cytometry* **13,** 137–143.

9

Monocyte/Macrophages and Dendritic Cells

Harry Segall and Richard E. Sutton

1. Introduction

Dendritic cells (DCs) are a heterogeneous population of bone marrow-derived antigen-presenting cells (APCs) that populate various lymphoid and nonlymphoid tissues, including the skin (Langerhans cells), lymph nodes (interdigitating and follicular DCs), spleen, and thymus. DCs are considered professional APCs, with a remarkable ability for initiating T-cell activation and the consequent immune response, but also for inducing tolerance ***(1–3)***. The regulation of these two distinct functions is not completely understood to date. Extensive recent studies have improved our understanding of DC development, differentiation, activation, and function. DCs exist as distinct subsets that differ in their lineage affiliation, surface molecule expression, and biological function. Each of these types of DCs can present antigens in either an immunogenic or tolerogenic manner according to their maturation state and antigen capture properties. Hence, DCs either induce the appropriate immune response to pathogens or prevent autoimmune reactivity, and could thus be useful for manipulating the immune system by cellular immunotherapy.

The powerful adjuvant activity that DCs possess in stimulating specific CD4 and CD8 T-cell responses has made them targets in vaccine development strategies for the prevention and treatment of infectious diseases and malignant processes, typically by transduction of DCs with genes encoding tumor or microbial antigens ***(4–7)***. Alternatively, DCs may be transduced with genes for chemokines, cytokines, or immunostimulatory proteins. Furthermore, the dual capacity to modulate the immune system uniquely positions DCs for the therapy of allograft rejection and autoimmune diseases ***(8,9)***. For instance, tolerogenic DCs could be generated to prolong graft survival by genetic

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engineering of donor- or recipient-derived DCs to express immunosuppressive molecules capable of promoting tolerance to alloantigen.

This extraordinary functional profile suggests that DCs may represent an ideal vector in the immunotherapy of cancer and infectious agents, as well as in graft rejection and autoimmunity, and one of the strategies being pursued to harness their potential is to transduce them with lentiviral vectors. Since lentiviral vectors have the capacity to transduce nondividing and terminally differentiated cells, they may be more suitable than other vectors for genetic modification of DCs, although careful vector comparisons are thus far lacking.

Dendritic cells are an APC population that is easily generated ex vivo using a variety of cytokines, which can also be used to further manipulate these cells in maturation and function. Human DCs may be derived from hematopoietic progenitors (CD34+-derived DCs) or from adherent peripheral blood monocytes (monocyte-derived DCs). Cultured DCs can be recognized by a typical veiled morphologic appearance and expression of surface markers that include major histocompatibility complex class II, CD86/B7.2, CD80/B7.1, CD83, and CD1a.

In the method presented here, peripheral blood mononuclear cells are first obtained by density gradient centrifugation. Monocytes are then isolated by plastic adherence. Dendritic cells can be obtained from monocytes by culturing with granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-4, and TNF-α.

2. Materials

2.1. Cell Lines

293T and human osteosarcoma (HOS) cells are available through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Rockville, MD). 293T cells are most commonly used for virus production by transient transfection of appropriate plasmids.

2.2. Maintenance of Cell Lines

HOS and 293T cells cultures are maintained in 10 mL complete Dulbecco's modified Eagle's medium (DMEM) in 10-cm tissue-culture dishes, in a 5% CO_2 water-jacketed incubator kept at 37°C. HOS cells are adherent and require trypsinization for passage. They are quite hardy, fast growing, and easily passaged at a 1:8 ratio twice weekly. 293T cells, which do not require trypsinization, should be passaged just prior to reaching confluence. Care should be taken so that they do not become overly confluent since that will reduce transfection efficiency. For maximally healthy cells a split of 1:3 or 1:4 of a 70–80% confluent cell culture plate every 3–4 d should provide optimal cell growth conditions.

2.3. Donor Peripheral Blood Mononuclear Cells (PBMCs)

Normal, healthy human donor buffy coats (heparinized, leukocyte-enriched whole blood fractions) may be obtained from a blood bank, typically for a small processing fee. These donors are screened by the standard assays currently required by the Food and Drug Administration (FDA) and the American Association of Blood Banks (AABB). Otherwise, whole blood may be obtained from patients by venous phlebotomy, with careful adherence to an institutional review board-approved protocol and applicable governing policies and procedures.

2.4. Reagents (All Sterile Unless Otherwise Indicated)

1. DMEM (GIBCO, Gaithersburg, MD, or equivalent).
2. Fetal bovine serum (FBS) (GIBCO or equivalent) (heat inactivated, 56°C, 30 min).
3. Penicillin-streptomycin (pen-strep) solution (GIBCO or equivalent). Concentration: 10,000 U Pen and 10 mg/mL Strep (considered 100X).
4. Ficoll-Paque (Pharmacia Biotech, Peapack, NJ).
5. Trypan blue: 0.4% (GIBCO or equivalent, does not need to be sterile).
6. Polybrene (Sigma, St. Louis, MO, or equivalent).
7. Dimethyl sulfoxide (DMSO) (Sigma or equivalent).
8. Chloroquine (Sigma), 1 m*M* prepared in dH_2O.
9. Trypsin-ethylenediaminetetraacetic acid (EDTA) solution: Trypsin 0.05%, EDTA 0.1% in phosphate-buffered saline (PBS).
10. Twelve-well flat bottom tissue-culture plates (Costar, Cambridge, MA).
11. 10-cm and 15-cm tissue-culture dishes and 150-mL tissue culture flasks (Costar).
12. Recombinant human GM-CSF, IL-4, and TNF-α (R & D Systems, Minneapolis, MN).
13. 0.01 *M* PBS, pH 7.2.
14. Purified DNA plasmids required for making replication-defective human immunodeficiency virus (HIV) vector. DNA is sterilized by standard salt-ethanol precipitation and resuspended in either sterile water or Tris-EDTA buffer, pH 8.0 at a concentration of 4 μg/mL.

2.5. Reagent Preparation

1. Complete medium: DMEM with 10% FBS and 1% pen-strep; store at 4°C.
2. Polybrene stock solution (5 mg/mL): dissolve 50 mg of polybrene into 10 mL PBS, filter-sterilize and store at 4°C. Aliquots are stable for at least 1 yr.

2.6. Additional Reagents for Calcium Phosphate Transfection

1. 2X HEPES-buffered saline (HBS): 16.4 g NaCl (0.28 *M* final), 11.9 g HEPES [N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (0.05 *M* final)], 0.21*g* Na_2HPO_4 (1.5 m*M* final), 800 mL H_2O. Adjust pH 7.05 with 5 *N* NaOH. Accurate pH and the amount of phosphate are crucial for optimal transfection. Add H_2O to 1 liter. Filter-sterilize and store at 4°C in aliquots.

2. 2.5 *M* $CaCl_2$: 183.7 g $CaCl_2$•$2H_2O$ (Sigma; tissue culture grade); H_2O to 500 mL. Filter-sterilize and store at 4°C in aliquots.
3. Sterile dH_2O.

3. Methods

3.1. Preparation of Virus Stocks by Calcium Phosphate Transfection

A precipitate containing calcium phosphate and DNA is formed by slowly mixing a HEPES-buffered saline solution with a solution containing calcium chloride and DNA. This precipitate is directly layered onto the cells and adheres to their surface. It should be visible by phase contrast or light microscopy 30 min after transfection. Up to 50% of the cells on a dish will take up the DNA precipitate. Shocking the cells with DMSO may improve transfection efficiency (*see* **Note 1**).

1. The day before transfection, seed 15-cm tissue culture dishes with 3×10^6 293T cells in 25 mL complete DMEM. We have found that the highest transfection efficiencies are obtained with 293T cells that are 50–70% confluent at the time of transfection. After adding cells, gently shake the plates forward and backward, then side to side, three to four times to distribute cells evenly on the plate. Set plates in incubator and allow cells to attach undisturbed overnight.
2. Prepare the DNA for application to cells. Cotransfect cells at room temperature with a mixture of 100 μg of each plasmid DNA per plate (e.g., transfer vector, packaging vector, and vesicular stomatitis virus G glycoprotein [VSVG] expression construct). Per 15-cm plate, add 100 μg DNA (of each plasmid), 0.125 mL 2.5 *M* $CaCl_2$, dH_2O to 1.25 mL. To the DNA-$CaCl_2$ mixture, add 1.25 mL of 2X HBS dropwise while mixing vigorously.
3. Distribute the precipitate solution evenly onto cells and gently agitate to mix precipitate and medium. Under a light microscope, you should observe evenly distributed small, dark particles.
4. Add chloroquine to each plate at a final concentration of 10 μ*M* and place cells back in incubator (*see* **Note 2**).
5. Incubate the cells 5–8 h under standard growth conditions. Remove the medium and gently add 5–10 mL of 10% DMSO in PBS, allow to sit for 1–2 min, then aspirate, and replace with 40 mL complete DMEM.
6. Harvest the vector supernatant 3 d posttransfection and remove cell debris by centrifugation at 2300*g* for 10 min. Supernatant can also be filter-sterilized at this point, if so desired.
7. Quantitate the number of transducing units per ml by titration on HOS cells. HOS cells are typically plated out the day before in 24-well format at 50,000 cells/well. Increasing amounts of vector supernatant are added (up to 0.1 mL) to each well and incubated overnight. The rest of the supernatant should be frozen at –80°C in appropriately sized aliquots. Cells are refed the following

day and examined for transgene expression 48–96 h later (depending on the nature of the transgene). Titer is back-calculated to 1 mL and should be between 5×10^5 and 5×10^6 IU/mL, depending upon the vector. Note in some cases the transgene may not be expressed in HOS cells and a more suitable target cell should be chosen for titering purposes.

3.2. Isolation of Mononuclear Cells from Peripheral Blood

Ficoll-Hypaque density gradient centrifugation is a simple and rapid method of purifying PBMCs that takes advantage of the density differences between mononuclear cells and other elements found in the blood sample. Mononuclear cells and platelets collect on top of the Ficoll-Hypaque layer because they have a lower density; in contrast, red blood cells (RBCs) and granulocytes have a higher density than Ficoll-Hypaque and collect at the bottom of the Ficoll-Hypaque layer. Platelets are separated from the mononuclear cells by subsequent washing. The monocytes are then separated from lymphocytes by adherence (*see* **Note 3**). Unless otherwise indicated, all procedures should be performed in a sterile manner.

1. Place fresh heparinized blood into 50-mL conical centrifuge tubes. Using a pipet, add an equal volume of room-temperature PBS. Mix well.
2. Slowly layer the blood/PBS mixture over the Ficoll-Hypaque solution. Use 20 mL Ficoll-Hypaque per 30 mL blood/PBS mixture. To maintain the Ficoll-Hypaque/blood interface, it is helpful to hold the centrifuge tube at a 45° angle.
3. Centrifuge for 30 min at 900*g* at room temperature without brake.
4. Using a pipet, remove the upper layer that contains the plasma and most of the platelets. Using another pipet, transfer the mononuclear cell layer to another centrifuge tube. Wash cells by adding excess PBS and centrifuging 10 min at 400*g*. Remove supernatant, resuspend cells in PBS, and repeat the wash once to remove most of the platelets.
5. Resuspend mononuclear cells in complete DMEM. Count cells and determine viability by Trypan blue exclusion.
6. If desired, determine the composition of the PBMC population by flow cytometry. Appropriate markers might include CD4, CD8, CD3, CD19, CD20, and CD14.

3.3. Isolation of Monocytes/Macrophages from Mononuclear Cells Using Adherence Method

Approximately 30–40% of the isolated mononuclear cells obtained are monocytes and macrophages. Monocytes may be isolated from the PBMC suspension by taking advantage of the fact that monocytes adhere to plastic, whereas lymphocytes do not.

1. Centrifuge mononuclear cells for 10 min at 300*g*. Remove supernatant and resuspend cell pellet in complete DMEM to a final concentration of 2×10^6 cells/mL. Transfer 50-mL cell suspension to a 150-cm^2 tissue culture flask.

2. Incubate horizontally for 1 h in a 37°C, 5% CO_2 humidified incubator.
3. Decant nonadherent lymphocytes. Rinse tissue culture flask gently three times with 37°C complete DMEM.
4. Add 50 mL complete DMEM.
5. Adherent cells can be recovered by vigorous washing with 0.02% EDTA in PBS and light scraping.
6. If desired, determine the purity of the monocyte population by flow cytometry, using CD14 as a marker.

3.4. Preparation of Dendritic Cells from Monocytes

1. Culture freshly prepared adherent monocytes at a density of 1×10^6 cell/mL in complete DMEM containing 800 U/mL recombinant GM-CSF and 500 U/mL recombinant IL-4. Feed the cells every 2 d by replacing the spent medium with freshly prepared medium.
2. On d 5, add 100 U/mL TNF-α. Continue to feed the cells every 2 d with medium containing all three cytokines.
3. Cells can be recovered by vigorous washing with 0.02% EDTA in PBS and light scraping.
4. By d 8, the cells should be fully mature dendritic cells, with characteristic veiled morphology and with at least 80% CD83-positive cells, as determined by flow cytometry (*see* **Fig. 1** and **Note 4**).

3.5. Transduction of Monocyte/Macrophages and Dendritic Cells

1. Immediately prior to transduction, thaw the required amount of vector stock at either room temperature or 37°C.
2. To monocyte or DC cultures at a concentration of $0.5–1 \times 10^6$ cells/mL in 24-well plates, 25-cm^2 tissue culture flasks, or 80-cm^2 tissue culture flasks, add appropriate amount of viral stock (*see* **Note 5**). Add polybrene to a final concentration of 4–8 μg/mL and incubate overnight at 37°C, 5% CO_2 in humidified incubator. Set up control cultures without added vector in each experiment.
3. Remove the vector supernatant from cultures by gently vacuum aspirating the medium without disturbing the cells, using a plastic aspirating pipet. Replace with fresh complete DMEM replete with all three cytokines for DC cultures. Feed cells every 2 d.
4. Three days after transduction, the percentage of transduced cells may be determined by flow cytometry, in the case of green fluorescent protein (GFP)- or yellow fluorescent protein (YFP)-encoding vectors (*see* **Fig. 1** and **Note 6**). Alternatively, transduced cells may be viewed by epifluorescence microscopy **(Fig. 2)**.

4. Notes

1. Optimal transfection of 293T cells requires high-quality plasmid DNA, prepared by CsCl ultracentrifuge purification or Qiagen ion-exchange column chromatography (Qiagen Inc., Valencia, CA). Construction and propagation of these plasmids is beyond the scope of this chapter, but many can be obtained from other workers in the field, and some are even commercially available (Invitrogen, Carlsbad, CA).

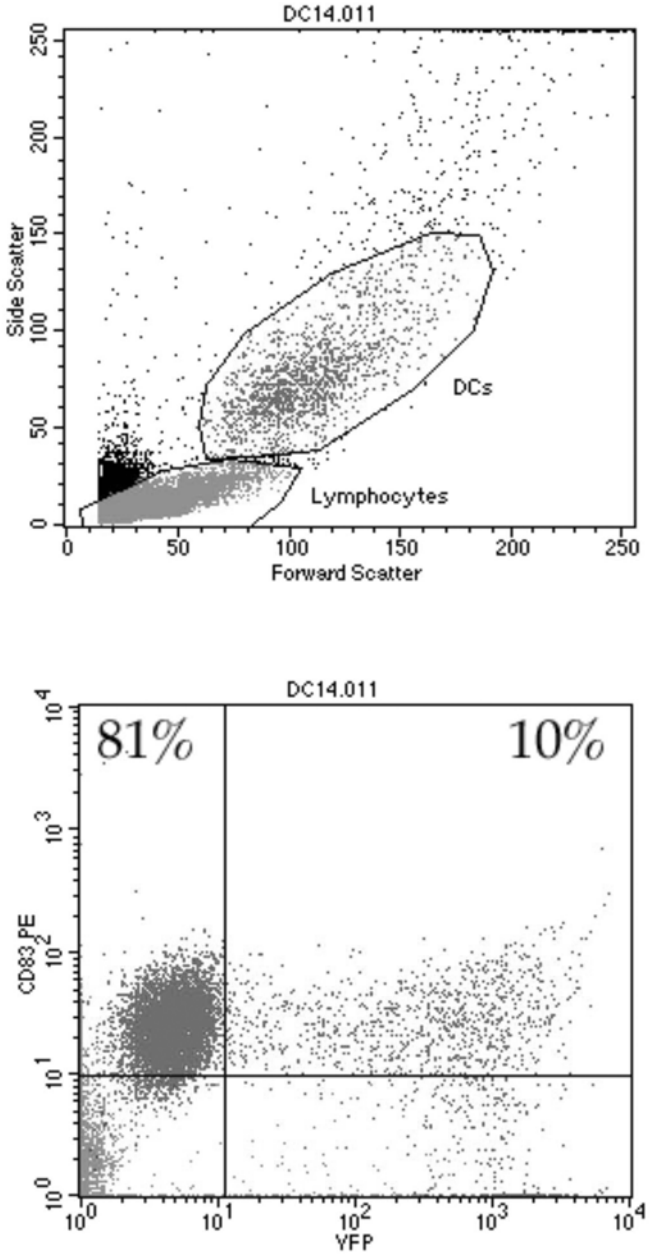

Fig. 1. Phenotype and transduction of DCs. At top is forward vs side scatter dot plot, with DCs gated. At bottom, note gated cells were mainly CD83+, and of those 11% were transduced by an eYFP-encoding HIV vector.

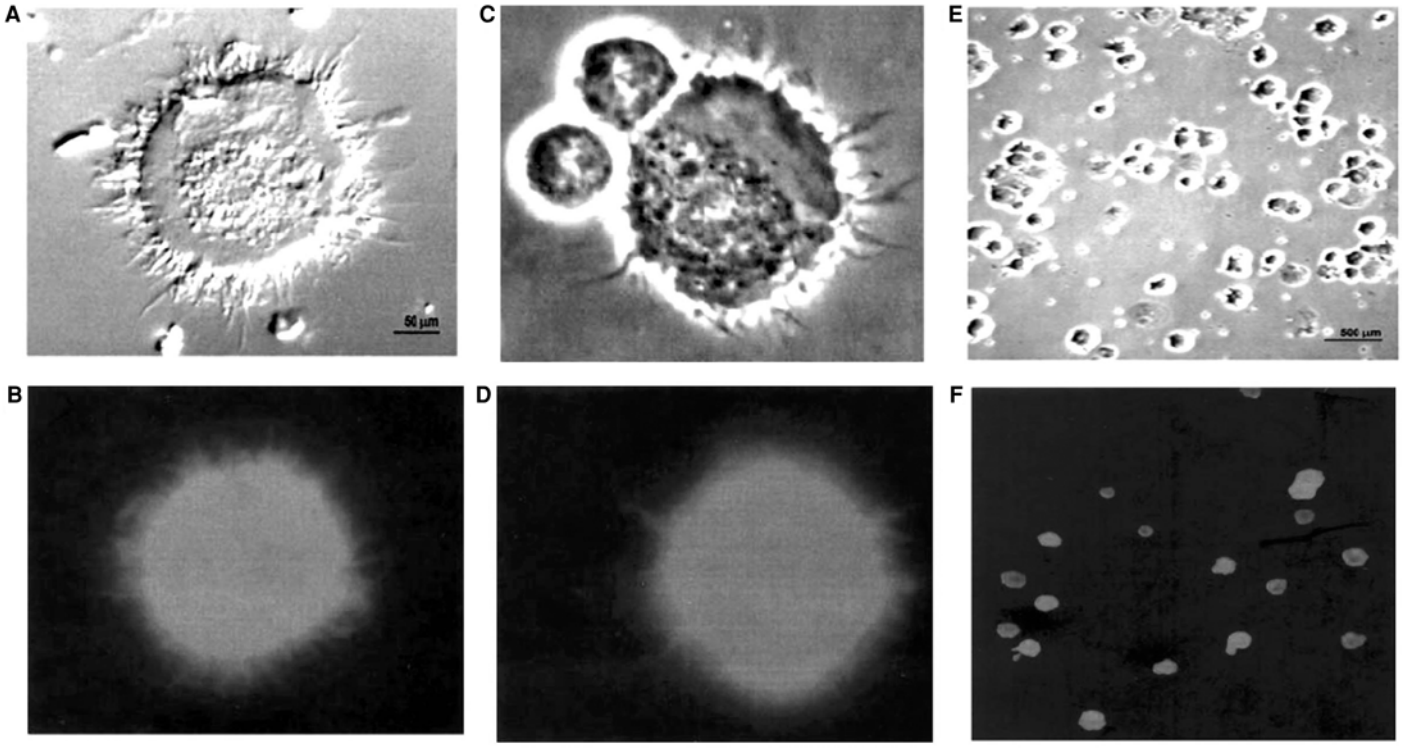

Fig. 2. Light microscopy of enhanced yellow fluorescent protein (eYFP)-transduced DCs, with phase-contrast **(A, C, E)** versus fluorescence images **(B, D, F)**. Reprinted with permission from Academic Press (Elsevier Science).

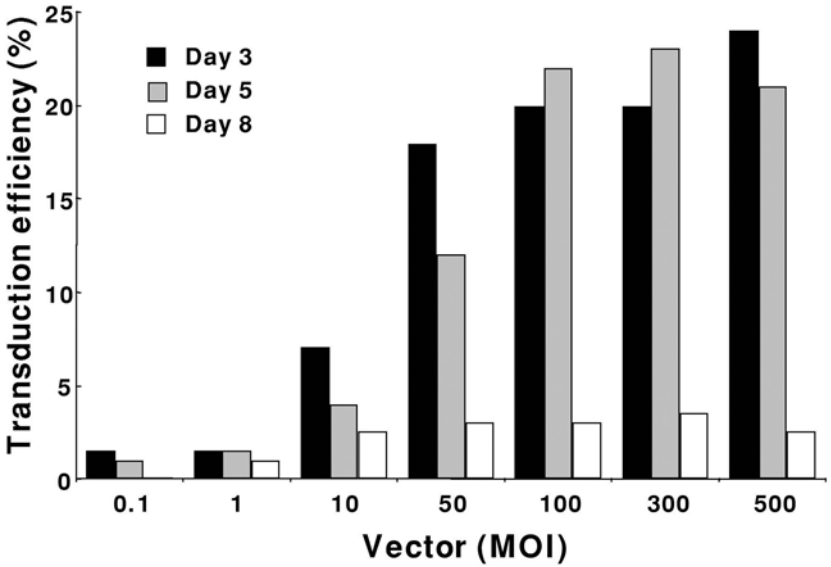

Fig. 3. Transduction efficiency of DCs is dependent on virus concentration and maturation status of DCs. MOI refers to multiplicity of infection and is proportional to vector titer. Reprinted with permission from Academic Press (Elsevier Science).

2. DNA delivered by calcium phosphate transfection is thought to transit through lysosomes to the nucleus, and chloroquine raises lysosomal pH, thus inhibiting lysosomal DNAses. Rigorous analysis suggests that chloroquine treatment (and the DMSO shock) is not strictly necessary, although refeeding the cells after 8 h is.
3. When working with human blood, cells, or infectious agents, universal precautions must be followed (irrespective of the fact that often the blood sample has been prescreened). For the use of this lentiviral vector system, Biosafety Level 2 practices with slight modification should be followed. In addition, all solutions and equipment coming into contact with cells must be sterile and proper sterile technique should be used accordingly.
4. Depending on the sample and culture conditions, sometimes 10–12 d are required to obtain mature DCs. Transduction efficiency is easily measured if an autofluorescent protein is present within the vector. If the vector does not encode a marker transgene, transduction efficiency may be measured by quantitative-competitive DNA PCR, using primers specific for the vector.
5. The amount of vector used depends upon the nature of experiment and how difficult it is to transduce the target population. If high transduction efficiencies are necessary, an ultracentrifuge-concentrated viral stock at a final concentration of at least $5 \times 10^7 - 5 \times 10^8$ IU/mL may be required.
6. We and others have observed a reduced transduction efficiency of mature DCs compared with monocytes or immature DCs **(Fig. 3)**. This reduction may reflect

a loss of endocytic ability with maturation, although postentry viral replication blocks have not been excluded. It is recommended to transduce the cells before maturation.

References

1. Lanzavecchia, A. and Sallusto, F. (2001) Regulation of T cell immunity by dendritic cells. *Cell* **106,** 263–266.
2. Liu, Y. J. (2001) Dendritic cell subsets and lineages and their functions in innate and adaptive immunity. *Cell* **106,** 259–262.
3. Mellman, I. and Steinman, R. M. (2001) Dendritic cells: specialized and regulated antigen processing machines. *Cell* **106,** 255–258.
4. Banchereau, J., Schuler-Thurner, B., Palucka, A. K., and Schuler, G. (2001) Dendritic cells as vectors for therapy. *Cell* **106,** 271–274.
5. Gunzer, M., Janich, S., Varga, G., and Grabbe, S. (2001) Dendritic cells and tumor immunity. *Semin. Immunol.* **13,** 291–302.
6. Sprinzl, G. M., Kacani, L., Schrott-Fischer, A., Romani, N., and Thumfart, W. F. (2001) Dendritic cell vaccines for cancer therapy. *Cancer Treat. Rev.* **27,** 247–255.
7. Steinman, R. M. and Dhodapkar, M. (2001) Active immunization against cancer with dendritic cells: the near future. *Int. J. Cancer* **94,** 459–473.
8. Ludewig, B., Junt, T., Hengartner, H., and Zinkernagel, R. M. (2001) Dendritic cells in autoimmune diseases. *Curr. Opin. Immunol.* **13,** 657–662.
9. Steinman, R. M. and Nussenzweig, M. C. (2002) Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc. Natl. Acad. Sci. USA* **99,** 351–358.

10

Hematopoietic Stem and Progenitor Cells

Robert E. Donahue and Irvin S. Y. Chen

1. Introduction

There has been much effort in isolating hematopoietic stem cells (HSC) and some confusion within the literature on what even a HSC is, with some investigators using the term inappropriately ***(1)***. A HSC is a cell capable of both self-renewal and of deriving progeny of all hematopoietic cell lineages, that is, a single HSC can regenerate erythroid, myeloid, megakaryocytic, and lymphoid lineages in a myeloablated host. Progenitor cells, on the other hand, are cells committed to one or a few lineages having a limited capacity for self-renewal. Progenitors are the progeny of HSC.

To target the HSC with a therapeutic gene has been the dream of many and continues to remain a dream to be realized. So far, no investigator has yet been able to document that a single cell can be transduced with a vector that is capable of reconstituting all hematopoietic lineages of a transplanted host. The reason for this failure is multifold. First, it is difficult to isolate a HSC. Investigators have generated multiple monoclonal antibodies in an effort to better define a HSC, and yet, no single monoclonal antibody has been found that recognizes a HSC exclusively. Many studies suggest that HSC reside in a cell population that expresses the CD34 antigen, a sialomucin protein that functions as a ligand for the L-selectin molecule ***(2)***. Initially CD34 was first identified on the human erythroleukemia cell line (KG-1a) ***(3)*** and later found to be expressed on vascular endothelium ***(4)*** as well as on presumptive HSC and their progeny. CD34 is expressed in 1–4% of human marrow cells, including all hematopoietic progenitor cells that can be detected using a variety of in vitro assays ***(5)***. Approximately 1% of those mononuclear cells isolated from cord blood ***(6–8)***, 0.2% from peripheral blood, and from 0.6%, 0.4%, and

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0.2% of mononuclear cells isolated from leukapheresis products acquired from patients receiving, respectively, chemotherapy alone, the cytokine granulocyte colony-stimulating factor (G-CSF) alone, or the combination of chemotherapy and G-CSF express CD34 ***(9,10)***. Cells expressing CD34 have been found to contribute to the reconstitution of both human ***(11)*** and nonhuman primate ***(12)*** hematopoiesis following transplantation. Other more recent studies, however, suggest that the presumptive HSC may not express CD34 ***(13,14)***, and thus, the whole subject remains a controversial topic ***(15)***. Other studies suggest another way of isolating the HSC. The HSC appears also to be a very distinctive population of cells capable of excluding the nucleic acid staining dye Hoechst 33342 ***(16,17)*** and are known as side population (SP) cells, which may or may not express CD34. In any case, both $CD34^+$ cells ***(4,18)*** and SP cells ***(19–21)*** are themselves heterogenous, with the presumptive HSC still representing only a fraction of these otherwise phenotypically well-defined populations of cells.

One physical attribute that many investigators agree that the HSC has is that it does not actively proliferate in vivo. This quiescence provides yet another obstacle in effectively transducing the HSC and should be considered seriously when selecting vector and culture conditions. The success of murine retroviral-mediated gene transfer, for example, is highly dependent on the proliferative status of the target cell. In order for murine retrovirus infection and integration to occur in the host cell's genome, cellular division is required ***(22,23)***, i.e., nuclear membrane breakdown is necessary for retroviral integration to occur. This makes efficient transduction of infrequently dividing cells with murine retroviral vectors problematic, requiring in many instances, the use of hematopoietic growth factors to stimulate replication of the target cell population in vitro. This, in turn, leads to still other difficulties, in that closely associated with proliferation is differentiation. Therefore, media, culture conditions and cytokine selection are critical in any transduction procedure. The ideal is to successfully elicit proliferation without subsequent differentiation, thereby allowing the target cell population to increase in numbers without further differentiation. Unfortunately this goal is hard to achieve. Cytokine and serum supplementation frequently induces primitive cells to differentiate, negatively affecting the potential efficacy of ex vivo $CD34^+$ cell gene therapy. In this context, lentivirus vectors may have a selective advantage over murine retroviruses in that not only are lentivirus-based vectors capable of transducing a cell population in a relatively short period of time (24 h or less) without active cell division, but also because transduction and subsequent transgene expression can be achieved in the absence of cytokines ***(24–28)***.

Still other obstacles remain even if a HSC can be successfully transduced with a vector. Critical amongst these is being able to document that the cell

can give rise to all hematopoietic lineages. A daunting task, especially as mature red blood cells and platelets are not nucleated and isolating progenitors and mature cells of defined lineages for integration analysis is difficult. New technologies and methodologies are being developed to allow such analysis to be performed on selected populations of cells ***(29,30)***. Ideally, therefore, the quest to successfully transduce primitive HSC involves (1) as precisely as possible to isolate and target a well-defined cell population containing the HSC, (2) to transduce the target cell population with a safe and efficacious vector that can successfully integrate within the target cell genome without disturbing the cell's function or capacity to differentiate, and (3) to have the inserted transgene be expressed at appropriate levels in the target cell population and in all of the target cell's progeny. To meet these requirements is a daunting task. The aim of this chapter is to provide guidelines to (1) isolate through immunoselection a target cell population that may contain the presumptive HSC and (2) describe a transduction method that has the potential to transduce this isolated cell population using a lentivirus vector.

2. Materials

1. Source Tissue (neonatal cord blood [CB], peripheral blood [PB], leukapheresis product [LP], bone marrow [BM]) should be preferably fresh; however, frozen mononuclear cells can also be used.
2. Phosphate-buffered saline (PBS) pH 7.4 without calcium or magnesium chloride (GIBCO BRL, Grand Island, NY). Stored at room temperature.
3. Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). Stored at room temperature.
4. Puregene red blood cell (RBC) lysis solution (Gentra Systems, Minneapolis, MN). Stored at room temperature.
5. 0.4%Trypan Blue Stain (GIBCO). Stored at room temperature.
6. Bovine serum albumin (BSA) Fraction V Solution 7.5% (GIBCO). Stored at 4°C.
7. 100-μm Nylon Cell Strainer (Becton Dickinson, Franklin Lakes, NJ).
8. Biotinylated $CD34^+$ antibody clone 12.8 (CellPro, Bothell, WA). Stored frozen in small aliquots at 1 mg/mL concentration until use.
9. Streptavidin MicroBeads (Miltenyi Biotec, Auburn, CA). Stored at 4°C.
10. 0.5 *M* ethylenediaminetetraacetic acid (EDTA) pH 8.0 (Molecular Biology Grade) (Quality Biological, Gaithersburg, MD). Stored at room temperature.
11. Magnetic cell sorting (MACS) Separation columns (Miltenyi Biotec). Kept at room temperature.
12. MACS Magnetic Device (Miltenyi).
13. Vector stock, concentrated or unconcentrated, frozen or fresh supernatant with a known multiplicity of infection (MOI) titer. The supernatant should be free of replication-competent virus, cellular debris, and potentially toxic or inhibitory proteins.

14. RetroNectin (BioWhittaker, Walkersville, MD, or TakaraShuzo Co., Shiga, Japan). Store at –20°C until use. Follow manufacturer's instructions for reconstitution.
15. Sterilized distilled water or Ultra Pure Water (Advanced Biotechnologies, Columbia, MD).
16. Millipore Millex-GV 0.22-μm filter (Millipore, Bedford, MA).
17. Six-well polystyrene nontissue culture-treated plate (Becton Dickinson).
18. Dulbecco's Modified Eagle Medium (DMEM) (GIBCO), plus 10% fetal calf serum (FCS) (Hyclone, Logan, Utah). Defined media that is serum free can be used as a substitute. One example is X-Vivo 10 (BioWhittaker).
19. 50 mg/mL Gentamicin Reagent Solution (GIBCO) 50 mg/mL stock.
20. In all instances sterile disposable plastic wear should be used and all appropriate Biosafety measures should be followed in performing the tissue culture procedures and handling the samples. All waste should be appropriately handled and disinfected.

3. Methods

3.1. Source of Hematopoietic Stem Cells

The source of tissue for isolation of primitive hematopoietic cells is clearly important (*see* **Note 1**). Hematopoietic stem cells are rare and represent only a small fraction of the cells present in BM and PB. In addition to adult tissues, neonatal CB and fetal liver have been used as sources of hematopoietic progenitors and stem cells. As neonatal CB is frequently discarded following birth and has been found to be a rich source of immature progenitors and potential hematopoietic stem cells, this source has become an increasingly popular reagent for both research and therapeutic purposes. The number of stem and progenitor cells recovered from both adult, newborn, and fetal tissue may be few. To increase the number of cells recovered, hematopoietic growth factors have been used successfully to mobilize hematopoietic stem and progenitor cells in circulating peripheral blood. Through the use of hematopoietic growth factors such as G-CSF and stem cell factor (SCF), collection of large numbers of immature cells has been achieved using leukapheresis procedures from cytokine mobilized peripheral blood (mPB). Use of mPB immunoselected $CD34^+$ cells therapeutically has accelerated the hematopoietic recovery of patients following myeloablative therapy. mPBs are the cells that are used in this particular description. The mPB leukapheresis product and BM typically contains heparin (10U/mL) and/or citrate (10mg/mL) to prevent aggregation and clotting.

3.2. Immunoselection (see Notes 2 and 3)

1. To perform the immunoselection procedure, dilute the LP from the mPB approx 1:1 with PBS at room temperature and gently layer 35 mL of this diluted

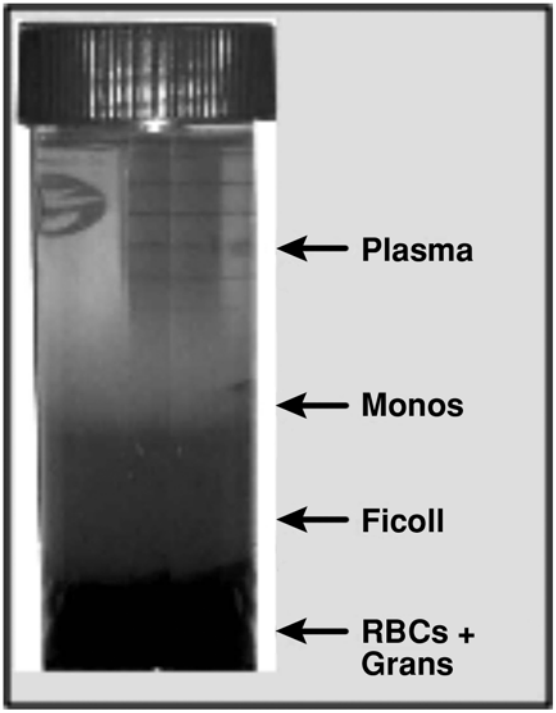

Fig. 1. Peripheral blood separation using Ficoll-Paque. Layers are granulocyte and red blood cells, Ficoll-Paque, mononuclear cells, and platelet rich plasma with PBS.

product on 15 mL of Ficoll-Paque Plus in a 50-mL polypropylene conical tube (Falcon, Becton Dickinson). (For smaller volumes 10 mL of diluted cells can be layered on 5 mL of Ficoll-Paque Plus in a 15-mL polypropylene conical tube) (*see* **Note 4**).

2. Centrifuge the cells at 400*g* at 20°C for 30 min with the mononuclear cell layer collected. The mononuclear cells are in the layer between the plasma and platelet layer (upper clear/cloudy layer) and the Ficoll-Paque layer (the Ficoll-Paque layer is immediately above the red blood cell and granulocyte pellet) **(Fig. 1)**.
3. Wash the collected mononuclear cell interface in PBS and, if there are contaminating RBC present, remove the RBC by ammonium chloride RBC lysis. RBC lysis is performed by resuspending the mononuclear cell pellet in a 50-mL volume of Puregene RBC lysis solution for 10 min at room temperature followed by centrifugation at 400*g* at 20°C for 10 min. This process can be repeated a second time should RBC contamination remain after the initial lysis step.
4. Following the RBC lysis, wash the cells in PBS and count using a hemocytometer using an appropriate dilution of 0.4%Trypan Blue Stain, a dye that evaluates

cell membrane integrity and, therefore, viability. Should the cell membrane be damaged, the cell stains blue and is considered nonviable.

5. Following the cell count, resuspend the mononuclear cells at approx 1–2 × 10^8/mL of 1% BSA in PBS and add the biotinylated primary antibody. The optimal concentration of antibody is dependent on the antibody used, so the manufacturer's instructions should be consulted. For the CellPro clone 12.8 biotinylated antibody, this concentration is approx 100 μL (1 mg/mL) per 1–5 × 10^9 cells. This particular clone is no longer commercially available, but other biotinylated CD34 antibodies are from Becton Dickenson PharMingen, San Diego, CA. Careful screening may be necessary to determine antibody specificity and avidity on cells of interest, especially if nonhuman primate tissues are being used.
6. Upon adding the antibody, incubate the cells at room temperature on a rocker for 30 min.
7. After the 30 min, wash the cells twice to remove any free, nonbound antibody and resuspend the cells at approx 1–2 × 10^8/mL in PBS plus 1% BSA along with the superparamagnetic Streptavidin MicroBeads. Use 2 mL of the colloidal suspension of Streptavidin MicroBeads per 1–5 × 10^9 cells.
8. Incubate the cells for an additional 30 min at room temperature on the rocker, wash twice, and resuspend in a separation buffer consisting of PBS with 0.5% BSA and 2 m*M* EDTA at a concentration of approx 2 × 10^8 cells/mL.
9. As the procedure described here is a positive selection, a LS+ MACS column is used for up to 10^8 positive cells. Again, the manufacturer's instructions should be followed in selecting which column to use. There are many types of columns, and the selection of column is dependent on number of cells to be collected and the type of collection (positive or negative). Typically we use multiple columns for a leukapheresis product, pooling the positively selected cells, and then passing the final product of $CD34^+$ cells through one last column **(Fig. 2)**.
10. Assess the purity of the immunoselection procedure by flow cytometry using an ELITE flow cytometer (Beckman Coulter, Miami, FL) after staining with an allophycocyanin (APC)-conjugated anti-CD34 monoclonal antibody (clone 563; a kind gift from Dr. Gustav Gaudernack, Institution of Transplantation Immunology, Rikshospitalet, The National Hospital, Oslo, Norway, and now commercially available through PharMingen) or another antibody that recognizes a distinct epitope from the one used for the positive selection in the immunoselection procedure. The purity of the immunoselected $CD34^+$ cells is routinely 90–95%.

3.3. Transduction of Immunoselected Cells (see Notes 5–7)

1. Coat nontissue culture-treated plates with fibronectin fragment CH-296 (RetroNectin) at a concentration of approx 50 μg/mL following reconstitution with sterile water, filtration with a 0.22-μm filter, and dilution with PBS. The concentration of RetroNectin to be used can vary and the instructions provided by the manufacturer should be followed accordingly (*see* **Note 8**).
2. Resuspend immunoselected $CD34^+$ cells in DMEM supplemented with 10% FCS or serum-free X-Vivo 10 and 50 μg/mL gentamicin sulphate at a concentration of

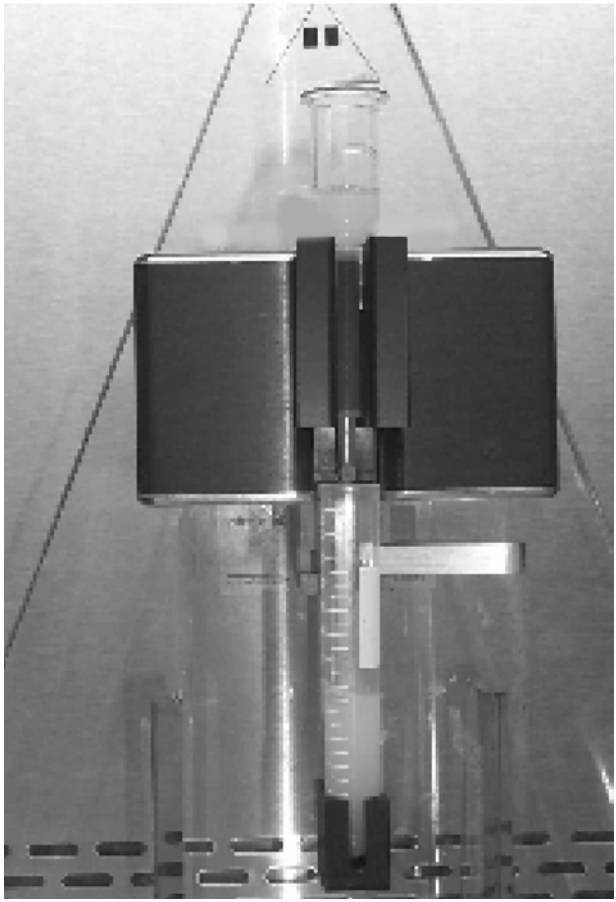

Fig. 2. Immunoselection using Miltenyi Biotech Magnetic cell sorting (MACS) device and columns. Cells are loaded on the column, with the CD34-biotin-labeled cells being retained on the column by bound streptavidin-coated paramagnetic particles and CD34-negative cells coming through the column in the eluate.

0.5 to 1 × 10^6 cells/mL. Incubate cells at 37°C in a 5% CO_2/95% air humidified environment on RetroNectin-coated (fragment CH-296, commercially available as a recombinant protein under the tradename RetroNectin), nontissue culture-treated 6-well plates.

3. Following an overnight incubation, transduce cells with a 40-fold dilution (the extent of dilution is dependent on the MOI desired. In our case, we prefer a low MOI of between 1 and 5. Others have used MOI of up to 100, but one needs to be cautious of toxicity of the frozen lentivirus stock).

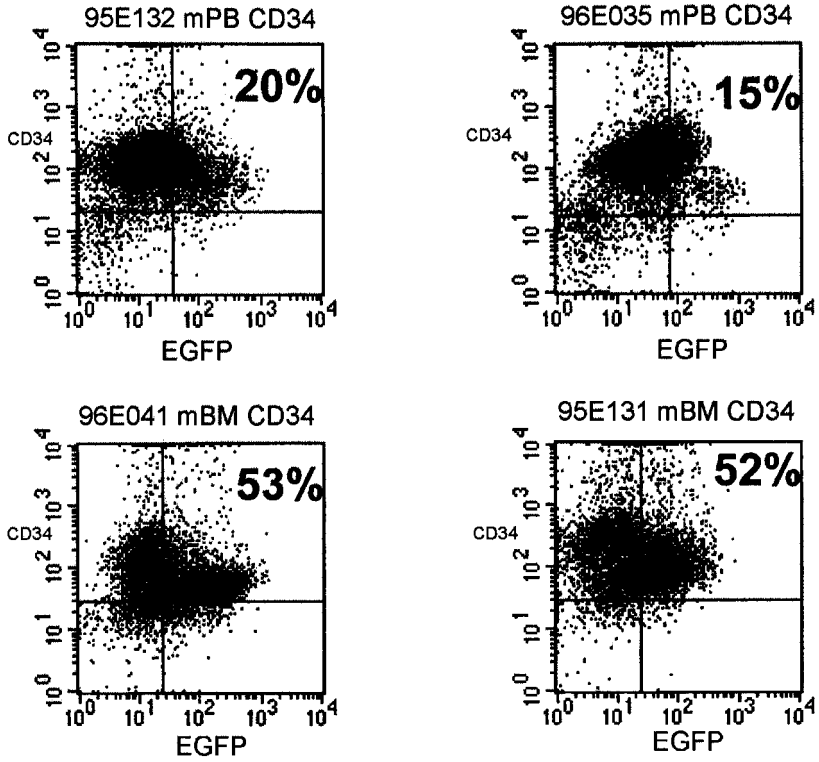

Fig. 3. EGFP expression of mBM and mPB purified CD34$^+$ cells 24 h following transduction with a lentiviral vector. CD34 expression using CD34-APC-labeled antibody and EGFP expression are evaluated.

4. Transductions can be performed either once or twice a day for up to 2 d at lower MOI with no evidence of toxicity.
5. Ideally a marker gene, such as the enhanced green fluorescent protein (EGFP), is incorporated within the vector to help determine transduction efficiency. EGFP expression can be measured by flow cytometry using a standard filter setup for fluorescein (525-nm bandpass filter) following transduction. Typically transgene expression with a lentiviral vector is apparent following a 24-h transduction and is independent of the inclusion of growth factors in the transduction media. This is in contrast to murine retroviral vectors, where little to no transgene expression is observed within the first 24 h of transduction. An example of EGFP expression following a 48-h transduction of immunoselected nonhuman primate CD34$^+$ cells from two cytokine mobilized bone marrow (mBM) immunoselections and two mPB immunoselections using a lentiviral vector is shown in **Fig. 3**.

4. Notes

1. The most frequent hematopoietic cell population isolated from BM, cord blood, and mobilized PB (mPB) for isolating hematopoietic stem and progenitor cells are those cells that express CD34. Following collection, the target cell population is identified and isolated as best as possible from contaminating cells. The rationale for purification is to (1) provide a defined population of cells for gene transfer, (2) eliminate any contaminating or accessory cells that may elicit differentiation or produce inhibitory signals, and (3) increase the vector-to-target cell ratio. A number of techniques have been developed to isolate $CD34^+$ cells from hematopoietic tissue. Counterflow centrifugation (elutriation) was used early on ***(31)***, as were more elaborate methods involving high-speed fluorescence-activated cell sorting. Cell sorting requires sophisticated instrumentation, utilizes an open system that can risk contamination of the product, may result in significant cell loss depending on the stringency of the sort criteria, and can require prolonged periods of time for collection of rare cell populations if no initial positive or negative selection is performed on the starting product. As cell sorting requires specialized instrumentation and is currently being used predominantly as a research tool, it will not be discussed further here. The method most frequently used for target cell selection is immunoselection using immunoadsorptive columns or immunomagnetic procedures.
2. There are several methods available for immunoselection of $CD34^+$ cells. One of the most common is in performing immunomagnetic separation using superparamagnetic beads. The method presented involves this technique using a biotinylated $CD34^+$ antibody, Streptavidin superparamagnetic microBeads, and MACS Separation columns. Immunoselection involves the use of an antibody or a collection of antibodies. Antibody selection is critical. Before proceeding the antibody should be titered and tested to confirm that it recognizes the target cell of interest.
3. The immunoselection itself can be positive or negative, depending on the approach taken and antibody selected. Positive selection, for example, is when the cell of interest is selected using an antibody that recognizes an antigen on the target-cell population. Negative selection, on the other hand, is when an antibody is selected to remove contaminating cell populations, thereby enriching the desired target-cell population. The method presented above is that of a positive selection. Negative selections follow a similar methodology, but columns of greater size are frequently used as greater numbers of cells are removed when enriching the product for the desired cell type. Should the immunoselection procedure require reinfusion of cells into a patient, Food and Drug Administration (FDA)-approved and licensed instrumentation will be required with the product to be reinfused tested for sterility and viral contaminants. The guidelines outlined above are simply just that and are meant for research purposes only.
4. As one scales up the procedure, problems do occur with cellular and platelet clumping. It is important to keep all reagents at room temperature, as cold

temperatures can lead to cellular aggregation. Should aggregation occur, or just as a precautionary step to prevent the clogging of a column, a cell strainer or filter can be used to remove aggregates prior to placing the cells over a column. This step is strongly suggested; otherwise purity of the final product can be affected. A 100-μm nylon filter can be used to help remove any cell aggregates.

5. After isolating the target cell population, the cells are ready to be transduced with an appropriate vector. Vector selection and titer are both critical if successful transduction is to be achieved. Initial pilot studies evaluating vector transduction efficiency at defined MOIs using differing targeting envelop proteins, packaging cell lines, and differing promoters and enhancers will prove useful in optimizing conditions prior to any large scale endeavor. Hematopoietic cell lines, such as KG-1a, Mo1E, and so on (American Type Culture Collection, Rockville, MD), can be used if primary cells are difficult to acquire. How vectors are constructed and titer determination will be discussed elsewhere.
6. After identifying a suitable vector candidate, transduction studies using primary cells can be performed. Ideally the vector selected expresses a clearly definable marker gene, such as enhanced green fluorescent protein (EGFP). Such marker genes permit rapid evaluation of protein expression in both nucleated and non-nucleated (such as RBC and platelets) cells.
7. Many lentivirus-based vectors have been described. Vectors based on the human immunodeficiency virus type 1 (HIV-1) have been used successfully to transduce cells from diverse organ systems, such as brain, muscle, and blood ***(32–35)***. Based on their ability to infect terminally differentiated cells or cells blocked in $G_{0/1}$, lentiviral vectors are thought to offer a selective advantage over other viral strategies for transgene delivery to primitive hematopoietic stem cells ***(36)***. Studies indicate, however, that target cells must be transcriptionally active for efficient infection by lentiviruses ***(37)***, corresponding to the G_{1b} phase of the cell cycle. The G_{1b} phase of the cell cycle is defined as cells with G_1 DNA content and having RNA levels equivalent to those observed in early S phase prior to DNA synthesis. Thus lentiviral vectors appear to be appropriate for transduction of primitive hematopoietic cells as well as other cell types that may not be actively proliferating. In addition, transduction of hematopoietic cells can be performed in the absence of additional hematopoietic growth factors, thereby minimizing the risk of differentiation of the immunoselected cells while in culture. As there are many different methods of transduction of a variety of cell types, clearly preliminary experimentation is necessary to optimize transduction conditions. The example used above is just one method for transducing immunoselected $CD34^+$ cells.
8. The use of fibronectin (FN)-treated plates in the transduction of the $CD34^+$ cells improves transduction efficiency in some instances and not others. Early studies demonstrated that the colocalization of target cells and retrovirus onto the extracellular matrix protein FN enhance viral transduction efficiencies. For example, gene transfer of mouse long-term reconstituting HSC and human HSC cells is improved following infection of cells in the presence of a chymotryptic

carboxy-terminal fragment of FN containing the high affinity heparin binding site and the binding site to integrin α4β1 ***(38,39)***. The inclusion of the binding sequence arg-gly-asp (RGD), which is recognized by the α5β1 integrin as a cell adhesion site in the FN fragment, further improved retrovirus-mediated gene transfer of murine reconstituting and human CD34+ HSCs (fragment CH-296) ***(40,41)*** and enhanced the frequency of genetically marked cells in the circulation of transplanted nonhuman primates following gene transfer ***(42)***. The mechanism by which matrix proteins, such as the intact FN molecule or a specific fragment, can enhance retrovirus-mediated gene is thought to be due in part to FN improving the physical geometry of the cell-virus interaction ***(39)***. When lentiviral transduction was performed on CH-296-coated surfaces for 24 or 48 h using mPB, consistent improvement in transduction efficiency were observed ***(28)***. For BM, however, differences between transduction efficiency between CH-296-coated and noncoated plates were not as apparent. This may be due potentially to differences in cell cycle kinetics, as BM- and mBM-derived $CD34^+$ cells appear to have a higher number of proliferating cells and may be more transcriptionally active than that of circulating mPB $CD34^+$ cells, thereby being more readily susceptible to transduction.

References

1. Orlic, D. and Bodine, D. M. (1994) What defines a pluripotent hematopoietic stem cell (PHSC): will the real PHSC please stand up! *Blood* **84,** 3991–3994.
2. Baumheter, S., Singer, M. S., Henzel, W., et al. (1993) Binding of L-selectin to the vascular sialomucin CD34. *Science* **262,** 436–438.
3. Civin, C. I., Brovall, C., Fackler, M. J., Schwartz J. F., and Shaper J. H. (1984) Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J. Immunol.* **133,** 157–165.
4. Fina, L., Molgaard, H. V., Robertson, D., et al. (1990) Expression of the CD34 gene in vascular endothelial cells. *Blood* **75,** 2417–2426.
5. Andrews, R. G., Singer, J. W., and Bernstein, I. D. (1990) Human hematopoietic precursors in long-term culture: single CD34+ cells that lack detectable T cell, B cell, and myeloid cell antigens produce multiple colony-forming cells when cultured with marrow stromal cells. *J. Exp. Med.* **172,** 355–358.
6. Bender, J. G., Unverzagt, K., Walker, D. E., et al. (1994) Phenotypic analysis and characterization of $CD34^+$ cells from normal human bone marrow, cord blood, peripheral blood, and mobilized peripheral blood from patients undergoing autologous stem cell transplantation. *Clin. Immunol. Immunopathol.* **70,** 10–18.
7. Sutherland, D. R., Keating, A., Nayar, R., Anania, S., and Stewart, A. K. (1994) Sensitive detection and enumeration of $CD34^+$ cells in peripheral and cord blood by flow cytometry. *Exp. Hematol.* **22,** 1003–1010.
8. Fritsch, G., Stimpfl, M., Buchinger, P., et al. (1994) Does cord blood contain enough progenitor cells for transplantation? *J. Hematother.* **3,** 291–298.

9. Bender, J. G., Unverzagt, K. L., Walker, D. E., et al. (1991) Identification and comparison of CD34-positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor flow cytometry. *Blood* **77,** 2591–2596.
10. Bender, J. G. and Unverzagt, K. (1993) Flow cytometric analysis of peripheral blood stem cells. *J. Hematother.* **2,** 421–430.
11. Berenson, R. J., Bensinger, W. I., Hill, R. S., et al. (1991) Engraftment after infusion of CD34+ marrow cells in patients with breast cancer or neuroblastoma. *Blood* **77,** 1717–1722.
12. Berenson, R. J., Andrews, R. G., Bensinger, W. I., et al. (1998) Antigen CD34+ marrow cells engraft lethally irradiated baboons. *J. Clin. Invest.* **81,** 951–955.
13. Osawa, M.., Hanada, K., Hamada, H., and Nakauchi, H. (1996) Long-term lymphohematopoietic reconstitution by a single CD34- low/negative hematopoietic stem cell. *Science* **273,** 242–245.
14. Sato, T., Laver, J. H., and Ogawa, M. (1999) Reversible expression of CD34 by murine hematopoietic stem cells. *Blood* **94,** 2548–2554.
15. Goodell, M. A. (1999) CD34+ or CD34–: Does it Really Matter? *Blood* **94,** 2545–2547.
16. Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., and Mulligan, R. C. (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* **183,** 1797–1806.
17. Goodell, M. A., Rosenzweig, M., Kim, H., et al. (1997) Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat. Med.* **3,** 1337–1345.
18. Gronthos, S., Franklin, D. M., Leddy, H. A., Robey, P. G., Storms, R. W., and Gimble, J. M. (2001) Surface protein characterization of human adipose tissue-derived stromal cells. *J. Cell Physiol.* **189,** 54–63.
19. Jackson, K. A., Mi, T., and Goodell, M. A. (1999) Hematopoietic potential of stem cells isolated from murine skeletal muscle. *Proc. Natl. Acad. Sci. USA* **96,** 14,482–14,486.
20. Jackson, K. A., Majka, S. M., Wang, H., et al. (2001) Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J. Clin. Invest.* **107,** 1395–1402.
21. McKinney-Freeman, S. L., Jackson, K. A., Camargo, F. D., Ferrari, G., Mavilio, F., and Goodell, M. A. (2002) Muscle-derived hematopoietic stem cells are hematopoietic in origin. *Proc. Natl. Acad. Sci. USA* **99,** 1341–1346.
22. Varmus, H. E., Padgett, T., Heasley, S., Simon, G., and Bishop, J. M. (1977) Cellular functions are required for the synthesis and integration of avian sarcoma virus-specific DNA. *Cell* **11,** 307–319.
23. Miller, D. G., Adam, M. A., and Miller, A. D. (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell Biol.* **10,** 4239–4242.
24. Bukrinsky, M. I., Haggerty, S., Dempsey, M. P., et al. (1993) A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature* **365,** 666–669.

25. Reiser, J., Harmison, G., Kluepfel-Stahl, S., Brady, R. O., Karlsson, S., and Schubert, M. (1996) Transduction of nondividing cells using pseudotyped defective high- titer HIV type 1 particles. *Proc. Natl. Acad. Sci. USA* **93,** 15,266–15,271.
26. Akkina, R. K., Walton, R. M., Chen, M. L., Li, Q. X., Planelles, V., and Chen, I. S. (1996) High-efficiency gene transfer into $CD34^+$ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *J. Virol.* **70,** 2581–2585.
27. An, D. S., Koyanagi, Y., Zhao, J. Q., et al. (1997) High-efficiency transduction of human lymphoid progenitor cells and expression in differentiated T cells. *J. Virol.* **71,** 1397–1404.
28. Donahue, R. E., Sorrentino, B. P., Hawley, R. G., An, D. S., Chen, I. S., and Wersto, R. P. (2001) Fibronectin fragment CH-296 inhibits apoptosis and enhances ex vivo gene transfer by murine retrovirus and human lentivirus vectors independent of viral tropism in nonhuman primate CD34+ cells. *Mol. Ther.* **3,** 359–367.
29. Schmidt, M., Hoffmann, G., Wissler, M., et al. (2001) Detection and direct genomic sequencing of multiple rare unknown flanking DNA in highly complex samples. *Hum. Gene Ther.* **12,** 743–749.
30. Kim, H. J., Tisdale, J. F., Wu, T., et al. (2000) Many multipotential gene-marked progenitor or stem cell clones contribute to hematopoiesis in nonhuman primates. *Blood* **96,** 1–8.
31. Jones, R. J., Wagner, J. E., Celano, P., Zicha, M. S., and Sharkis, S. J. (1990) Separation of pluripotent hematopoietic stem cells from spleen colony-forming cells. *Nature* **347,** 188, 189.
32. Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996a) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. USA* **93,** 11,382–11,388.
33. Naldini, L., Blomer, U., Gallay, P., et al. (1996b) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.
34. Reiser, J., Harmison, G., Kluepfel-Stahl, S., Brady, R. O., Karlsson, S., and Schubert, M. (1996) Transduction of non-dividing cells using pseudotyped defective high- titer HIV type 1 particles. *Proc. Natl. Acad. Sci. USA* **93,** 15,266–15,271.
35. Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H., and Verma, I. M. (1997) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat. Genet.* **17,** 314–317.
36. Sutton, R. E., Wu, H. T., Rigg, R., Bohnlein, E., and Brown, P. O. (1998) Human immunodeficiency virus type 1 vectors efficiently transduce human hematopoietic stem cells. *J. Virol.* **72,** 5781–5788.
37. Korin, Y. D. and Zack, J. A. (1998) Progression to the G1b phase of the cell cycle is required for completion of human immunodeficiency virus type 1 reverse transcription in T cells. *J. Virol.* **72,** 3161–3168.
38. Moritz, T., Patel, V. P., and Williams, D. A. (1994) Bone marrow extracellular matrix molecules improve gene transfer into human hematopoietic cells via retroviral vectors. *J. Clin. Invest.* **93,** 1451–1457.

39. Moritz, T., Dutt, P., Xiao, X., et al. (1996) Fibronectin improves transduction of reconstituting hematopoietic stem cells by retroviral vectors: evidence of direct viral binding to chymotryptic carboxy-terminal fragments. *Blood* **88,** 855–862.
40. Hanenberg, H., Xiao, X. L., Dilloo, D., Hashino, K., Kato, I., and Williams, D. A. (1996) Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat. Med.* **2,** 876–882.
41. Hanenberg, H., Hashino, K., Konishi, H., Hock, R. A., Kato, I., and Williams, D. A. (1997) Optimization of fibronectin-assisted retroviral gene transfer into human $CD34^+$ hematopoietic cells. *Hum. Gene Ther.* **8,** 2193–2206.
42. Kiem H. P., Andrews R. G., Morris J., et al. (1998) Improved gene transfer into baboon marrow repopulating cells using recombinant human fibronectin fragment CH-296 in combination with interleukin-6, stem cell factor, FLT-3 ligand, and megakaryocyte growth and development factor. *Blood* **92,** 1878–1886.

11

Mesenchymal Stem Cells

Xian-Yang Zhang, Vincent F. La Russa, and Jakob Reiser

1. Introduction

Adult bone marrow contains both hematopoietic and nonhematopoietic stem cells, also termed mesenchymal stem cells (MSCs), or mesenchymal progenitor cells. MSCs are cells capable of differentiating into cells of the mesenchymal lineage ***(1–3)***. These cells were previously referred to as "marrow stromal cells" or "colony-forming unit-fibroblast" (CFU-F), reflecting their origin and morphology in culture ***(4,5)***. Bone marrow stroma is the main source of these multipotent nonhematopoietic mesenchymal stem cells ***(6,7)***. MSCs derived from bone marrow can be readily isolated and expanded in vitro. Due to their ability of self-renewal and their potential to differentiate into terminal osteocytes, chondrocytes, myocytes, tenocytes, adipocytes, and neural cells in vivo and in vitro ***(8–11)***, bone marrow-derived MSCs have attracted considerable attention as potential tools for therapeutic gene transfer.

A variety of studies using different viral vectors have attempted to transduce MSCs ***(12–15)***. Due to their capacity to integrate into the host genome, the use of retroviral vectors has been widely investigated. This investigation has led to the successful expression of numerous genes including reporter genes such as *Escherichia coli* β-galactosidase ***(14–16)*** and enhanced green fluorescent protein (EGFP) ***(17,18)***, as well as many therapeutic genes including coagulation factors VIII ***(19–21)*** and IX ***(22–24)***, interleukins 3 ***(15,16,25)*** and 7 ***(26)***, human growth hormone ***(22)***, human erythropoietin ***(27)***, arylsulfatase A ***(28,29)***, tyrosine hydroxylase GTP cyclohydrolase I ***(30,31)***, and α-L-iduronidase ***(32)***. However, gene transfer into MSCs using oncogenic retroviruses is limited overall due to a low efficiency of transduction and a general lack of long-term transgene expression, probably caused by promoter inactivation.

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We attempted to optimize human immunodeficiency virus type 1 (HIV-1) based lentiviral vectors for efficient delivery and expression of transgenes in human bone marrow-derived stromal cells ***(33)***. Our results indicate that a single round of transduction using unconcentrated HIV-1-based vectors pseudotyped with vesicular stomatitis virus G glycoprotein (VSV-G) can lead to the efficient transduction of human MSCs. Transduction efficiencies and expression levels with HIV-1-derived lentiviral vectors were found to be higher than those with murine stem cell virus-based vectors ***(34)*** pseudotyped with VSV-G, indicating that HIV-1-based lentiviral vectors are superior to vectors based on oncogenic retroviruses as far as MSCs are concerned. Stable expression of reporter genes up to at least 10 months was observed. Clonogenic mesenchymal progenitor cells could also be transduced by HIV-1-based lentiviral vectors, with transgene expression being maintained by their mesenchymal progeny cells over several cell divisions and during differentiation into adipocytes, providing evidence that the capacity of differentiation of the cells was unaffected by lentivirus-mediated reporter gene transfer ***(33)***. In this chapter, we present protocols describing the establishment of bone marrow-derived MSC cultures and the application of HIV-1-derived lentiviral vectors to deliver efficiently transgenes into cultured MSCs. These protocols provide practical tools for using lentivirus-mediated gene transfer strategies for ex vivo modification of MSCs.

2. Materials

It is recommended that all cell culture work be carried out using disposable plasticware and that proper sterile measures be taken. All lentivirus containing waste should be treated with hypochlorite and then autoclaved.

1. Heparinized syringes, precoated with M-199 medium (Sigma, St. Louis, MO) containing 100 U/mL preservative-free heparin.
2. Heparin (American Pharmaceutical Partners, Inc., Los Angeles, CA).
3. Dulbecco's phosphate-buffered saline (DPBS, without calcium chloride and magnesium chloride, GIBCO/Invitrogen, Carlsbad, CA).
4. Ficoll (Lymphocyte Separation Media, density 1.077 g/mL, ICN, Aurora, OH).
5. Dimethyl sulfoxide (DMSO, Cryoserv, Edwards Lifesciences, Irvine, CA).
6. Cryopreservation medium: M-199 medium (Sigma) containing 30% selected fetal bovine serum (FBS) (Hyclone, Logan UT, *see* **Note 1**), 10% DMSO.
7. Trypsin-ethylenediaminetetraacetic acid (EDTA) solution (GIBCO/Invitrogen).
8. Trypan Blue stain (0.4%, GIBCO/Invitrogen).
9. Long-term culture medium (LTCM): Iscove's modified Dulbecco's medium (IMDM) supplemented with 5×10^{-6} *M* hydrocortisone (Sigma), 2.5 m*M* L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all from GIBCO/

Invitrogen), and 25% selected equine serum (Hyclone, *see* **Note 1**). The medium was filtered though a 0.45-μm filter, stored at 4°C and used within 7 d.
10. Phosphate-buffered saline (PBS, GIBCO/Invitrogen).
11. Polybrene (Hexadimethrine bromide, Sigma): 8 mg/mL in H_2O, sterilized by passing through a 0.2-μm filter unit. Store aliquots at –20°C.
12. Hank's balanced salt solution (GIBCO/Invitrogen) supplemented with 2% FBS.
13. Formaldehyde (PolySciences, Warrington, PA), 2% in PBS.
14. Crystal Violet (Sigma), 0.2 % in 20% ethanol.
15. Dibutyryl cyclic adenosine 5′ monophosphate (dbcAMP, Sigma): 200 m*M* in H_2O, sterilized by passing through a 0.2-μm filter unit. Store aliquots at –20°C.
16. Isobutylmethylxanthine (IBM-X, Sigma), 100 m*M* in ethanol. Store aliquots at –20°C.

3. Methods

3.1. Preparation of Cryopreserved Human Bone Marrow Low-Density Cells (LDCs)

Human bone marrow specimens should be obtained from healthy volunteers (*see* **Note 2**) and collected using heparinized syringes.

1. Dilute bone marrow sample (1 : 10) with DPBS containing 10 U/mL of heparin.
2. Load cell suspension (15 mL) slowly over 15 mL Ficoll in a 50-mL conical centrifuge tube.
3. Centrifuge at 150*g* for 30 min at room temperature using a swinging bucket rotor (IEC centrifuge, Needham Heights, MA). Do not engage brake.
4. Recover marrow low density (<1.07 g/mL) mononuclear cells carefully from interphase and resuspend in up to 30 mL of DPBS containing 10 U/mL heparin. Centrifuge for 10 min at 150*g* at room temperature.
5. Resuspend cell pellet in cryopreservation medium at a density of 20×10^6 nucleated marrow cells per mL (*see* **Note 3**).
6. Aliquot 1 mL marrow cells per Nunc cryovial and cryopreserve cells using a controlled rate freezer (Planer Kryo 10 Series III, TS Scientific, Perkasie, PA). Store the frozen vials in liquid nitrogen.

3.2. Preparation of Human Bone Marrow Derived Mesenchymal Stromal Cell Cultures

1. Rapidly thaw cryopreserved human bone marrow cells in a 37°C water bath.
2. Suspend cells in a total of 10 mL of LTCM (*see* **Note 4**) supplemented with 10 U/mL heparin. Count nucleated cells with a hemocytometer (*see* **Note 5**).
3. Plate cells in T150 flasks at a density of 1×10^7 viable nucleated marrow cells in 30 mL of LTCM per flask. Maintain cells in a humidified incubator at 37°C and 5% CO_2.

4. Feed cultures weekly by completely removing all nonadherent cells and spent medium and replacing it with 30 mL fresh medium (*see* **Note 6**). Keep the culture for a total of 16 to 18 d to allow colony formation (*see* **Note 7**).
5. Wash the cells twice with 10 mL of PBS and add 3 mL of Trypsin-EDTA per flask and incubate at 37°C for 3 min to detach completely cells. Add 6 mL of LTCM to dissociate cell clumps and transfer entire cell suspension into a conical centrifuge tube.
6. Centrifuge at 150*g* for 5 min at room temperature. Resuspend cell pellet in 5 mL LTCM and count total number of nucleated cells.
7. Plate 1×10^5 stromal cells in 2 mL of LTCM to each well of 6-well plates for subsequent virus transduction (*see* **Note 8**).

3.3. Preparation of Lentiviral Vectors (see Note 9)

Details regarding lentivirus preparation are described in previous chapters of this book.

3.4. Transduction of MSCs

1. Sixteen hours after plating (*see* **Subheading 3.2.**), remove medium and replace with 0.5 mL of LTCM containing 8 μg/mL of polybrene and lentiviral vectors at various multiplicities of infection (MOIs). Include mock transduction as control using the same conditions but without virus. Return to 37°C incubator for 20 h.
2. Remove transduction medium and replace with 2 mL fresh LTCM.
3. Harvest MSCs 72 h after transduction and analyze by Fluorescence-Activated Cell Sorting (FACS) (*see* **Subheading 3.5.**) to determine expression of transgenes. Alternatively, keep transduced MSCs in LTCM for desired time to observe long term transgene expression and differentiation (*see* **Subheading 3.6.**).

3.5. Flow Cytometric Analysis of Transgene Expression

1. Harvest transduced MSCs by adding 0.5 mL of Trypsin-EDTA per well of a 6-well plate.
2. Transfer MSCs to a tube and centrifuge at 500*g* for 8 min at 15°C to pellet cells.
3. Wash cell pellets twice with 2 mL Hank's solution.
4. Resuspend in 200 μL of Hank's solution and analyze by FACS (FACScan, Becton Dickinson, San Jose, CA). For analyzing the expression of the red fluorescent protein (DsRed) reporter gene, excite cells at 488 nm and detect emission in the FL2 channel at 575–605 nm (*see* **Note 10**).

3.6. Long-Term Maintenance and Induction of Differentiation of Transduced MSCs

1. Three days after transduction, trypsinize MSCs from the plate and replate cells at a density of 4×10^5 cells in 8 mL of LTCM per 100-mm culture dish.

2. Replace medium every 2 wk and split cells monthly. Analyze the cells by FACS and replate remaining cells (*see* **Note 11**).
3. Observe differentiation of terminal adipocytes using an inverted and/or fluorescence microscope (*see* **Note 12**).
4. To induce neural differentiation, incubate transduced MSCs in LTCM containing dbcAMP (1 m*M*) and IBM-X (0.5 m*M*) for 9 d beginning 72 h after transduction. Change medium every 3 d. Observe morphological changes of transduced cells using an inverted and/or fluorescence microscope (*see* **Note 13**).

3.7. Transduction of Clonogenic Mesenchymal Progenitor Cells

1. Thaw cryopreserved bone marrow cells and plate into 60-mm culture dishes at a density of 2.5×10^5 nucleated cells per culture dish containing 4 mL medium.
2. Twenty-four hours later, remove medium and nonadherent cells and replace with 1.5 mL of fresh medium containing 8 μg/mL polybrene and lentiviral vectors at various MOIs. Perform mock transductions using the same conditions but without virus.
3. After incubation at 37°C for 20 h, remove virus and replace with 4 mL of LTCM. Keep cells in the plates for two more weeks with medium changes at weekly intervals.
4. Two weeks after transduction, count the number of proliferating colonies that are emerging from transduced clonogenic mesenchymal progenitors under a fluorescence microscope. Count the total number of CFU-Fs per plate under an inverted microscope after staining with Crystal Violet (*see* **Note 14**). Analyze cells from parallel plates by FACS.

4. Notes

1. It is important to screen serum lots. Fetal bovine serum lots should be screened for viability and optimal growth of fresh and cryopreserved hematopoietic progenitor cells. Screen equine sera for mesencymal/stromal progenitor cell colony stimulation activity. Select the one with highest activity and reserve corresponding lot.
2. Consent of using human blood specimens must be approved and proper biosafety measures must be taken into account.
3. Nucleated cells are counted with a hemocytometer and viability determined by Trypan Blue dye exclusion.
4. It is very important that only freshly prepared medium be used for starting the culture and refeeding. This procedure will ensure proper proliferation of progenitor cells. We routinely prepare fresh medium on a weekly basis.
5. The viability of total nucleated cells in each specimen after thawing is typically around 70%.
6. On d 7 after replacement of medium, small clusters of adherent cells should be visible under a phase contrast microscope. The number of cells in each cluster varies from a few cells up to 30 cells. Colonies of strongly adherent fibroblast-

like cells are macroscopically visible after 2 wk of culture and are similar in appearance to those also derived from CFU-Fs. The colonies vary in size and are consisted with either uniform fibroblastlike cells or mixed cell populations including small blastlike cells, small endotheliallike cells, large fibroblastic cells, and adipocytes. Secondary cultures derived from these pooled 16–18 d colonies were used for transduction.

7. The yields of MSCs (after 16–18 d in culture) are in the range of 15–25% of all viable nucleated marrow cells plated. The variation in the yields is possibly due to the freshness of culture medium and the handling of cells.
8. For in vivo transplantation experiments, 5×10^5 cells in 8 mL medium per culture dish are transduced and harvested 3 d later.
9. The lentiviral vectors used in our laboratory are derived from the NL4-3 molecular clone ***(35)*** and contain the central PPT sequence ***(36,37)*** placed 5′ to the Rev responsive elements (RRE) sequence as described ***(38,39)***. Lentiviral vector particles are produced in 293T cells by transient cotransfection using a three-plasmid expression system ***(40)***. The titer (termed infectious units i.u./mL) of unconcentrated lentiviral vector stocks are routinely above 4×10^7 i.u./mL assayed with HOS cells (American Type Culture Collection, CRL-1543).
10. Based on FACS analysis, the efficiencies of transduction of human MSCs (determined by the percentage of EGFP positive cells) by lentiviral vectors encoding an EGFP gene are approx 5- to 10-fold lower than in other cell lines such as HOS cells. However, we found that the expression of the DsRed reporter gene in MSCs was much higher than in HOS cells or 293T cells ***(33)***. DsRed therefore provides an excellent alternative to EGFP in MSCs and its spectrum is not confounded by autofluorescence ***(41)***.

    The expression of transgenes (other than reporter genes encoding fluorescent proteins) in transduced MSCs can be assessed using specific antibodies. After washing with Hank's solution, incubate cells with phycoerythrin (PE) or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies on ice for 30 min, wash twice with Hank's solution, and resuspend in 100 μL PBS. Fix cells by adding 100 μL of 2% formaldehyde solution for 5 min and subject to FACS analysis.
11. We have maintained transduced MSCs in culture for up to 10 mo to assess the long-term expression of the EGFP and DsRed transgenes. FACS analysis profiles indicated that there were no significant changes in the percentage of positive cells. However, the mean fluorescence values were slightly reduced ***(33)***. Typically, EGFP and DsRed are stably expressed in transduced MSCs as long as the cells survive in culture.
12. The LTCM culture medium promotes differentiation of MSCs to terminal adipocytes. When undifferentiated MSC cultures were transduced with lentiviral vectors and maintained in LTCM for 1 mo, the formation of adipocytes with neutral lipid vacuoles could be directly assessed under a phase contrast microscope. Fluorescence microscopy confirmed that these adipocytes were derived from virus-transduced MSCs ***(33)***.

13. Induction of differentiation of transduced MSCs into neural cells was tested by treatment of cells with dbcAMP and IBM-X. The reagents had been shown to induce differentiation of marrow stromal cells into early progenitors of neural cells ***(42)***. Morphological changes were first observed 3 d after applying the inducers to transduced MSCs. By 9 d, up to 45% positive cells with neuronal appearance consisting of bipolar or multipolar cells with small cell bodies and elongated processes could be detected. Immunocytochemical analyses showed that monoclonal antibody against the neuron-specific antigen NeuN reacted with these MSC-derived neuronlike cells (Zhang, X.-Y., unpublished data).
14. There should be no difference in the average number and size of the mixed fibroblast/adipocyte colonies in virus-transduced or mock-transduced progenitor cells at low MOIs.

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References

1. Deans, R. J. and Moseley, A. B. (2000) Mesenchymal stem cells: biology and potential clinical uses. *Exp. Hematol.* **28,** 875–884.
2. Bianco, P., Riminucci, M., Gronthos, S., and Robey, P. G. (2001) Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells* **19,** 180–192.
3. Minguell, J. J., Erices, A., and Conget, P. (2001) Mesenchymal stem cells. *Exp. Biol. Med.* **226,** 507–520.
4. Castro-Malaspina, H., Gay, R. E., Resnick, G., et al. (1980) Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. *Blood* **56,** 289–301.
5. Friedenstein, A. J., Gorskaja, J. F., and Kulagina, N. N. (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp. Hematol.* **4,** 267–274.
6. Prockop, D. J. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276,** 71–74.
7. Conget, P. A. and Minguell, J. J. (1999) Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J. Cell Physiol.* **181,** 67–73.
8. Pittenger, M. F., Mackay, A. M., Beck, S. C., et al. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284,** 143–147.
9. Gerson, S. L. (1999) Mesenchymal stem cells: no longer second class marrow citizens. *Nat. Med.* **5,** 262–264.
10. Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., et al. (2000) Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp. Neurol.* **164,** 247–256.
11. Pereira, R. F., Halford, K. W., O'Hara, M. D., et al. (1995) Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc. Natl. Acad. Sci. USA* **92,** 4857–4861.

12. Frolova-Jones, E. A., Ensser, A., Stevenson, A. J., Kinsey, S. E., and Meredith, D. M. (2000) Stable marker gene transfer into human bone marrow stromal cells and their progenitors using novel herpesvirus saimiri-based vectors. *J. Hematother. Stem Cell Res.* **9,** 573–581.
13. Conget, P. A. and Minguell, J. J. (2000) Adenoviral-mediated gene transfer into ex vivo expanded human bone marrow mesenchymal progenitor cells. *Exp. Hematol.* **28,** 382–390.
14. Ding, L., Lu, S., Batchu, R., III, R. S., and Munshi, N. (1999) Bone marrow stromal cells as a vehicle for gene transfer. *Gene Ther.* **6,** 1611–1616.
15. Lee, K., Majumdar, M. K., Buyaner, D., Hendricks, J. K., Pittenger, M. F., and Mosca, J. D. (2001) Human mesenchymal stem cells maintain transgene expression during expansion and differentiation. *Mol. Ther.* **3,** 857–866.
16. Allay, J. A., Dennis, J. E., Haynesworth, S. E., et al. (1997) LacZ and interleukin-3 expression in vivo after retroviral transduction of marrow-derived human osteogenic mesenchymal progenitors. *Hum. Gene Ther.* **8,** 1417–1427.
17. Marx, J. C., Allay, J. A., Persons, D. A., et al. (1999) High-efficiency transduction and long-term gene expression with a murine stem cell retroviral vector encoding the green fluorescent protein in human marrow stromal cells. *Hum. Gene Ther.* **10,** 1163–1173.
18. Jaalouk, D. E., Eliopoulos, N., Couture, C., Mader, S., and Galipeau, J. (2000) Glucocorticoid-inducible retrovector for regulated transgene expression in genetically engineered bone marrow stromal cells. *Hum. Gene Ther.* **11,** 1837–1849.
19. Chuah, M. K., Brems, H., Vanslembrouck, V., Collen, D., and Vandendriessche, T. (1998) Bone marrow stromal cells as targets for gene therapy of hemophilia A. *Hum. Gene Ther.* **9,** 353–365.
20. Chiang, G. G., Rubin, H. L., Cherington, V., et al. (1999) Bone marrow stromal cell-mediated gene therapy for hemophilia A: in vitro expression of human factor VIII with high biological activity requires the inclusion of the proteolytic site at amino acid 1648. *Hum. Gene Ther.* **10,** 61–76.
21. Chuah, M. K., Van Damme, A., Zwinnen, H., et al. (2000) Long-term persistence of human bone marrow stromal cells transduced with factor VIII-retroviral vectors and transient production of therapeutic levels of human factor VIII in nonmyeloablated immunodeficient mice. *Hum. Gene Ther.* **11,** 729–738.
22. Hurwitz, D. R., Kirchgesser, M., Merrill, W., et al. (1997) Systemic delivery of human growth hormone or human factor IX in dogs by reintroduced genetically modified autologous bone marrow stromal cells. *Hum. Gene Ther.* **8,** 137–156.
23. Gordon, E. M., Skotzko, M., Kundu, R. K., et al. (1997) Capture and expansion of bone marrow-derived mesenchymal progenitor cells with a transforming growth factor-beta1-von Willebrand's factor fusion protein for retrovirus-mediated delivery of coagulation factor IX. *Hum. Gene Ther.* **8,** 1385–1394.
24. Cherington, V., Chiang, G. G., McGrath, C. A., et al. (1998) Retroviral vector-modified bone marrow stromal cells secrete biologically active factor IX in vitro and transiently deliver therapeutic levels of human factor IX to the plasma of dogs after reinfusion. *Hum. Gene Ther.* **9,** 1397–1407.

25. Nolta, J. A., Hanley, M. B., and Kohn, D. B. (1994) Sustained human hematopoiesis in immunodeficient mice by cotransplantation of marrow stroma expressing human interleukin-3: analysis of gene transduction of long-lived progenitors. *Blood* **83,** 3041–3051.
26. Bolotin, E., Smogorzewska, M., Smith, S., Widmer, M., and Weinberg, K. (1996) Enhancement of thymopoiesis after bone marrow transplant by in vivo interleukin-7. *Blood* **88,** 1887–1894.
27. Bartholomew, A., Patil, S., Mackay, A., et al. (2001) Baboon mesenchymal stem cells can be genetically modified to secrete human erythropoietin in vivo. *Hum. Gene Ther.* **12,** 1527–1541.
28. Matzner, U., Harzer, K., Learish, R. D., Barranger, J. A., and Gieselmann, V. (2000) Long-term expression and transfer of arylsulfatase A into brain of arylsulfatase A-deficient mice transplanted with bone marrow expressing the arylsulfatase A cDNA from a retroviral vector. *Gene Ther.* **7,** 1250–1257.
29. Matzner, U., Schestag, F., Hartmann, D., et al. (2001) Bone marrow stem cell gene therapy of arylsulfatase A-deficient mice, using an arylsulfatase A mutant that is hypersecreted from retrovirally transduced donor-type cells. *Hum. Gene Ther.* **12,** 1021–1033.
30. Schwarz, E. J., Alexander, G. M., Prockop, D. J., and Azizi, S. A. (1999) Multipotential marrow stromal cells transduced to produce L-DOPA: engraftment in a rat model of Parkinson disease. *Hum. Gene Ther.* **10,** 2539–2549.
31. Schwarz, E. J., Reger, R. L., Alexander, G. M., Class, R., Azizi, S. A., and Prockop, D. J. (2001) Rat marrow stromal cells rapidly transduced with a self-inactivating retrovirus synthesize L-DOPA in vitro. *Gene Ther.* **8,** 1214–1223.
32. Baxter, M. A., Wynn, R. F., Deakin, J. A., et al. (2002) Retrovirally mediated correction of bone marrow-derived mesenchymal stem cells from patients with mucopolysaccharidosis type I. *Blood* **99,** 1857–1859.
33. Zhang, X.-Y., La Russa, V. F., Bao, L., Kolls, J., Schwarzenberger, P., and Reiser, J. (2002) Lentiviral vectors for sustained transgene expression in human bone marrow-derived stromal cells. *Mol. Ther.* **5,** 555–565.
34. Cheng, L., Du, C., Murray, D., et al. (1997) A GFP reporter system to assess gene transfer and expression in human hematopoietic progenitor cells. *Gene Ther.* **4,** 1013–1022.
35. Adachi, A., Gendelman, H. E., Koenig, S., et al. (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* **59,** 284–291.
36. Follenzi, A., Ailles, L. E., Bakovic, S., Geuna, M., and Naldini, L. (2000) Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat. Genet.* **25,** 217–222.
37. Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., and Charneau, P. (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101,** 173–185.
38. Reiser, J. (2000) Production and concentration of pseudotyped HIV-1-based gene transfer vectors. *Gene Ther.* **7,** 910–913.

39. Reiser, J., Lai, Z., Zhang, X. Y., and Brady, R. O. (2000) Development of multigene and regulated lentivirus vectors. *J. Virol.* **74,** 10,589–10,599.
40. Soneoka, Y., Cannon, P. M., Ramsdale, E. E., et al. (1995) A transient three-plasmid expression system for the production of high titer retroviral vectors. *Nucleic Acids Res.* **23,** 628–633.
41. Matz, M. V., Fradkov, A. F., Labas, Y. A., et al. (1999) Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat. Biotechnol.* **17,** 969–973.
42. Deng, W., Obrocka, M., Fischer, I., and Prockop, D. J. (2001) In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. *Biochem. Biophys. Res. Commun.* **282,** 148–152.

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Lentivirus Gene Engineering Protocols

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12

Hippocampal Neurons

Jay L. Nadeau

1. Introduction

It has proven notoriously difficult to express foreign genes in primary neuronal cells in vitro. Some success has been achieved with lipid-mediated plasmid DNA transfection ***(1)***, transduction via mechanical methods (e.g., gene gun) ***(2,3)***, and infection with a variety of neurotropic viral vectors: adeno-associated virus (AAV) ***(4)***, Semliki Forest virus (SFV) ***(5)***, measles virus ***(6)***, Sindbis virus ***(7)***, and lentivirus ***(8)***. Of these methods, lentiviruses show the longest survival of infected neurons (up to 6 wk), with the least associated cytopathology. However, expression is not seen for at least 48 h postinfection, and expression levels are much lower than those resulting from Sindbis, SFV, and adenovirus. This makes lentiviruses ideal vectors for experiments in which effects of low levels of foreign gene expression are studied over long-term and less desirable for protein production or massive overexpression. We have found lentivirus impractical for acute slice recordings, as the preparations do not live long enough to demonstrate visible expression of reporter genes (e.g., green fluorescent protein [GFP]); for these cultures, AAV or SFV is preferable. In organotypic and dissociated cultures, however, neurons may be infected at any age in vitro, with a high rate of success (70 to >90% for dissociated cultures) **(Fig. 1)**.

Certain precautions particular to cultured systems are necessary to achieve these high transduction rates. Difficulties with infection are seen in culture that do not occur in vivo. Injections of human immunodeficiency virus (HIV)-based vectors into rat brain have reported that nearly 89% of cells infected are terminally differentiated neurons ***(9)***; however, in dissociated cultures, glial cells are always infected more readily than neurons **(Fig. 2A–C)**. The glia

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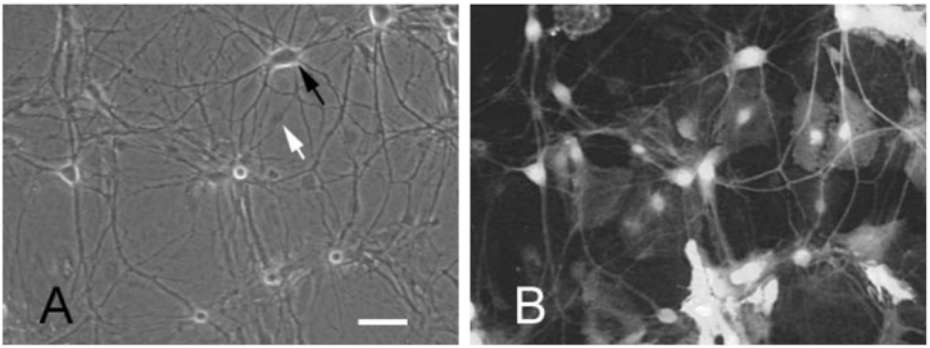

Fig. 1. Lentiviral vectors mediate high-efficiency expression in cultured hippocampal neurons. Scale bar = 20 μm. **(A)** Phase contrast image of lentivirus-GFP infected dish (21 d in culture [dic], 7 d postinfection [dpi]) showing neurons as three-dimensional cells surrounded by bright haloes (black arrow). Nuclei of glial cells (white arrow) are less readily observed and appear flat. **(B)** Fluorescence image. All neurons in this field of view show GFP expression, as do many of the glial cells.

are a mixed population of differentiated astrocytes of varying morphologies ***(10)***, oligodendrocytes ***(11)***, microglia, and others; they spread out to form a continuous underlayer with individual cells often more than 100 μm across. These large, actively dividing cells provide an ideal target for retroviruses. Timing of infection, virus concentration, and type of culture used can all be optimized in order to infect efficiently neurons rather than glia.

Desired levels of gene expression, survival times, and visibility vary from experiment to experiment. In addition, in vitro experiments often require lower concentrations and lower absolute viral counts than do in vivo injections. We attempt to make this protocol as versatile as possible by beginning with viral construct, and following the infection process through to the final use of the culture, be it electrophysiology, live imaging, or fixation and staining. When specific methods have not yet been tested, we make an effort to identify this fact and also to point out techniques we have tried that did not work.

1.1. Viral Constructs

The greatest disadvantage of low-level expression systems such as lentivirus is that lack of visible reporter gene expression does not necessarily mean a cell is uninfected. This is especially true of bicistronic vectors, in which a visible reporter such as GFP is expressed after an internal ribosomal entry site (IRES) or cap-independent translation enhancer (CITE) (*see* **Note 1**). Such a sequence recruits ribosomes directly to structured regions of mRNA upstream of the initiation codon and permits transcription from two sites independently **(Fig. 3A)**.

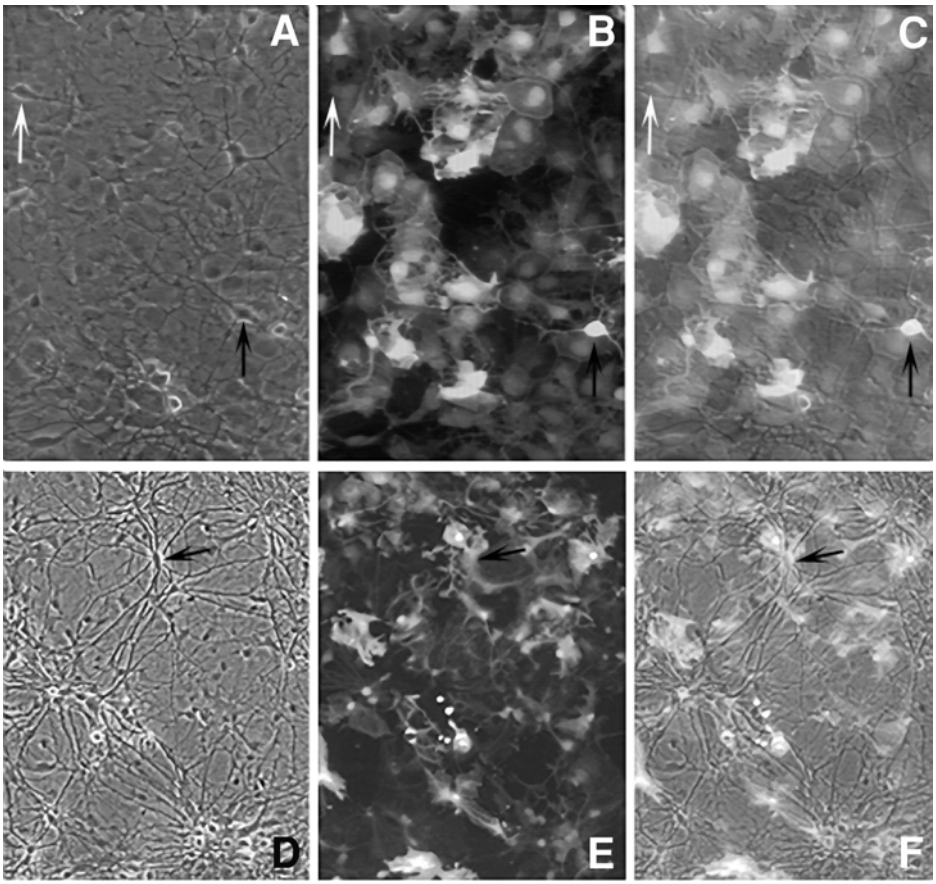

Fig. 2. Glial cells are readily infected and may obscure the neuronal signal. Scale bar = 20 μm. **(A**,**B**,**C)** Phase contrast, epifluorescence, and overlay of a GFP-infected culture (14 dic, 4 dpi). Some neurons sit far enough away from the glial bed that they either clearly do (black arrow) or do not (white arrow) express GFP; for many other cells, the situation is more ambiguous. **(D**,**E**,**F)** A similar situation is seen in a culture 10 dic, 9 dpi; early infection, before extensive growth of glia, does not prevent excessive glial labeling.

The level of expression of the gene following the IRES is often much lower than of the gene preceding it. However, such dual-gene vectors remain the best method for expressing a gene of interest and an independent visible reporter, and levels of correlation between the two genes of a bicistronic message are excellent ***(12)*** (*see* **Note 2**).

The woodchuck responsive element (WRE) from the woodchuck hepatitis virus functions as an enhancer when placed 3′ to the gene(s) of interest **(Fig. 3B)**.

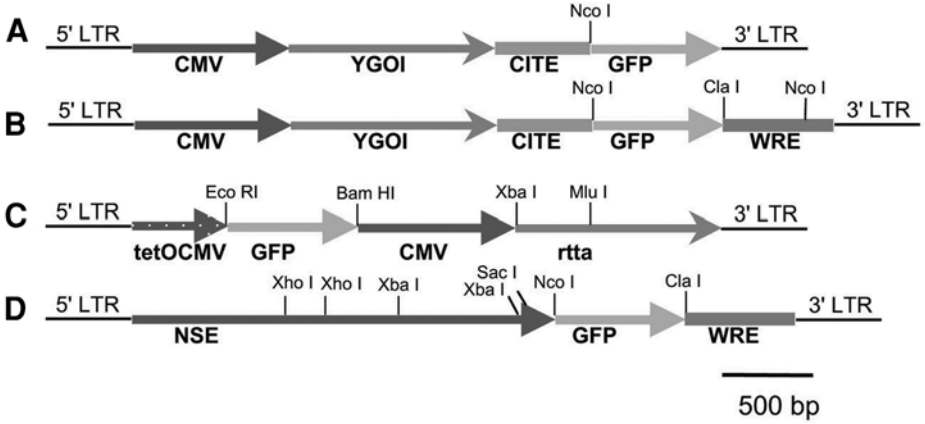

Fig. 3. Samples of bicistronic constructs for expression of a GFP reporter along with your gene of interest (YGOI). The entire cloned sequence falls between the HIV long terminal repeats (LTRs) in the vector pHR′ ***(28)***. **(A)** The GFP gene should always follow the CITE, and it must be in frame (the *Nco* I site provides a convenient means to do this). **(B)** The WRE, if desired, follows both genes. **(C)** A tetracycline-inducible construct showed moderate levels of inducibility. **(D)** The 2.2-kb NSE promoter fragment that has driven neuron-specific expression in other experiments did not work well in lentiviral vectors.

This 500-bp sequence increases lentiviral expression five- to eightfold ***(13)*** and leads to enhanced GFP production from both monocistronic and bicistronic constructs. GFP becomes visually detectable 24–48 h earlier with (Gene of Interest)-CITE-GFP-WRE than with (Gene of Interest)-CITE-GFP, and at 3–4 d postinfection shows a 2.5 to 3-fold increase in whole-cell fluorescence ***(13a)***. We recommend this enhancer for all bicistronic constructs where it enhances visibility without compromising survival.

However, the use of the enhancer in a monocistronic construct with GFP alone may lead to GFP toxicity ***(14)***. Neurons infected with monocistronic GFP-WRE show normal electrophysiological properties for only approx 7 to 9 d before they die (Nadeau and Lester, unpublished data; $n > 50$ cells); β-galactosidase (LacZ)-WRE demonstrates no harmful effects. A new, humanized GFP which may prove less toxic is available (Stratagene, La Jolla, CA, "vitality GFP"), though we have no data with this construct in neurons.

Several newer lentiviral constructs exist, among them self-inactivating and tetracycline-inducible vectors. The reverse tetracycline transactivator (rtta) construct shown in **Fig. 3C** ***(15)*** (reagents from Clontech, Palo Alto, CA) showed approximately fivefold induction with doxycycline, but high background levels (Nadeau and Lester, unpublished data, $n > 50$ cells); other con-

structs with the forward transactivator (tta) or with nuclear localization signals may improve inducibility ***(16)***. In experiments involving chronic exposure to tetracycline, it is worthwhile to note that the drug has behavioral effects on developing mice and rats ***(17)*** and may show toxicity to neurons in culture.

An attempt was made in our lab to create a neuron-specific lentivirus, driven by the neuron-specific enolase (NSE) promoter **(Fig. 3D)**. This fragment has driven expression in transgenic mice and in adenovirus ***(18)***, adeno-associated virus ***(19)***, and herpesvirus ***(20)*** vectors. However, its use in lentivirus was unsuccessful for us. Packaging cells displayed some fluorescence, but infected neurons did not show visible GFP. Future refinements of the construct may yield success.

1.2. Viral Harvest and Concentration

Extreme levels of concentration (1000- to 10,000-fold, resulting in titers in excess of 10^9) are not necessary for in vitro transduction. Sufficient titers for efficient infection of dissociated cultures can be obtained using filter units, which allow for 30- to 100-fold concentrations in 10 min or less. Although this is the easiest method, it is not always the best. If an ultracentrifuge is available, the preferred method of concentration is a single round of ultracentrifugation followed by complete draining of the pellet and resuspension in 1/200th to 1/500th of the original volume. This method results in the least viral loss, the most reliable infections, and the ability to infect organotypic slices as well as dissociated cells.

1.3. Culture System and Age In Vitro

Any type of hippocampal culture is amenable to lentiviral infection. However, ordinary, serum-containing "cocultures" of neurons and glia will demonstrate greater transduction of glial cells than differentiated neurons, as discussed in the Introduction and in Fig. 2. Embryonic cultures (E16 to E18) are preferable to postnatal cultures, as there are fewer glial cells. Infecting the neurons directly after plating does not work to eliminate glial labeling, as the few glia that exist at that time continue to divide, passing the lentiviral transgene to their progeny **(Fig. 2D–F)**. On the other hand, these cultures are robust and relatively resistant to excitotoxic injury and aging. We have infected such cultures anywhere from 1 h before plating to 8 wk in vitro with equal rates of success and long-term survival.

Other types of cultures may provide better results for imaging or other studies in which labeled glia present a problem.

1. Banker cultures. Such cultures consist of a glia-bearing coverslip inverted over a dish of serum-free neurons, so that the glial cells contact the neurons (*see* **Note 3**). Neurons are infected after the glia are removed **(Fig. 4A**,**B)**.

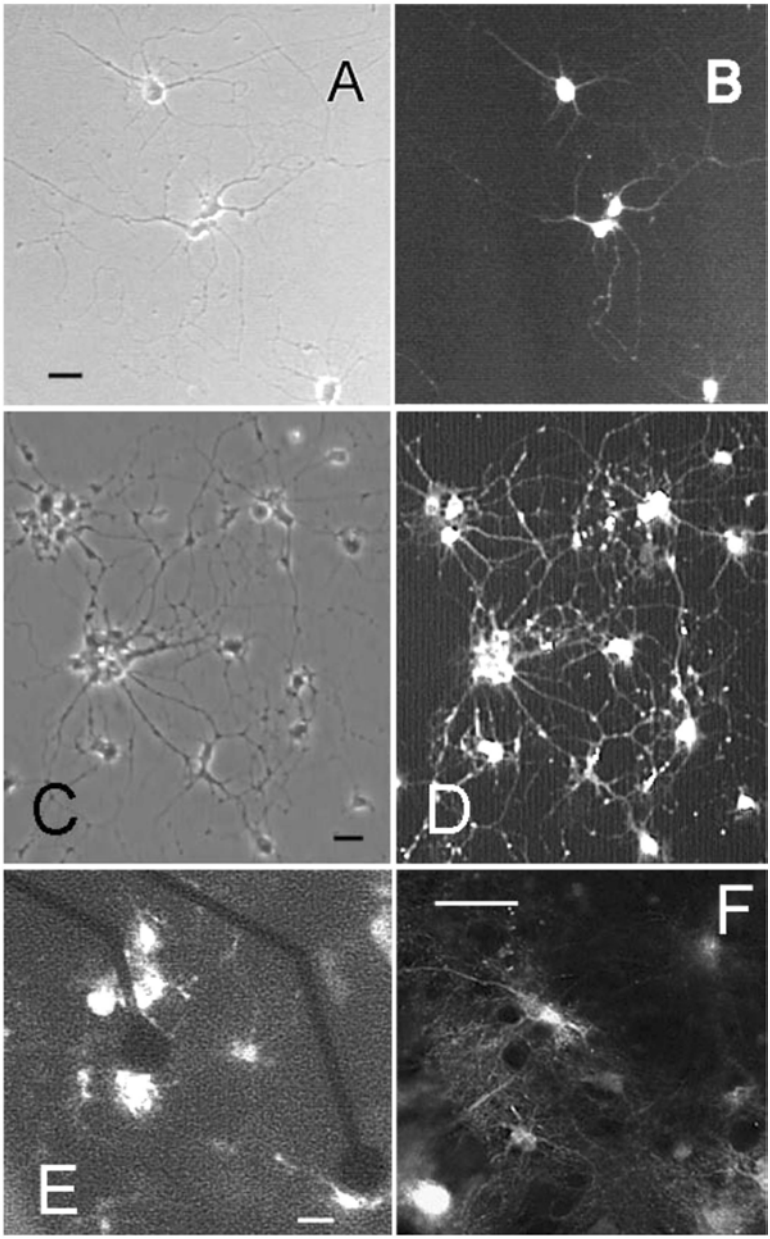

Fig. 4. Appearance of lentiviral infections of different hippocampal culture types. Scale bar = 20 μm. **(A**,**B)** Phase contrast and fluorescence images of a Banker culture, 14 dic, 12 dpi. The glial coverslip was removed when infection took place; the neurons remain healthy nearly 2 wk later. Serum-free culture conditions. **(C**,**D)** Serum-free culture without a glial coverslip, 10 dic, 9 dpi. The lack of glial cells makes the fluorescent neuronal processes clearly visible. **(E)** A multielectrode array, 6 dic, 6 dpi. Black circles are the electrodes. Fluorescent neurons are found both close to and far from electrodes. **(F)** fluorescent neurons in the CA3 layer of an organotypic slice culture (21 dic) after injection of approx 1 μL of concentrated lentivirus.

2. Serum-free cultures. These cultures show important physiological differences from cocultures (reduced cyclic adenosine monophosphate [cAMP] and ion channel differences *[21,22]*) and are more susceptible to excitotoxicity. Lower viral concentrations should be used. Results are often beautiful **(Fig. 4C,D)**.
3. Multielectrode arrays *(23)*. These are extremely fragile due to a lack of supporting glial cells. One-quarter to one-half of the usual viral amount should be used, and it should be washed out of the solution. Cells are not likely to live for more than 2–3 wk after infection **(Fig. 4E)**.
4. Organotypic slices. Bathing the slice in virus results in large numbers of labeled glia, but few or no neurons. Direct injection into the cell body layer is recommended **(Fig. 4F)**.

1.4. Reporter Genes

Choice of a reporter gene is an important question in experiments with living and fixed cultures. LacZ is extremely sensitive and provides excellent estimates of infection efficiency, but cannot be assayed without sacrificing the culture. GFP and its derivatives, blue fluorescent protein (BFP), yellow fluorescent protein (YFP), and cyan fluorescent protein (CFP), as well as the unrelated red fluorescent protein (DsRed, Clontech), remain the best visible markers in living cells; GFPs targeted to plasma membrane, actin filaments, nuclei, and mitochondria are all commercially available (Clontech; Invitrogen, Carlsbad, CA) and work well in neuronal culture.

2. Materials

2.1. Viral Harvest and Concentration

1. 293T packaging cells, 48–96 h posttransfection. It is advisable to wash the cells with fresh medium 8–12 h before harvest, as acidification of the medium destroys viruses.
2. Tris-buffered saline (TBS) with $MgCl_2$: 100 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 10 m*M* $MgCl_2$ (filter through 0.2-μm filter; stable at 4°C).
3. 60-mL syringes and 0.45-μm syringe filters (important: 0.2-μm filters will remove viruses).

2.1.1. Ultrafiltration

Centricon filter units, 100,000 MW cutoff (Millipore, Bedford, MA) (*see* **Note 4**).

2.1.2. Ultracentrifugation

1. A swinging-bucket rotor is essential to concentrate the virus into the bottom of the tube.
2. Ultracentrifuge tubes. We have had success with both 30-mL and 12-mL capacity. A harvest from two or three 150-mm plates will fill a rotor consisting of 6 12-mL tubes. For in vitro experiments, viral preps rarely need to exceed this size.

2.2. Reporter Genes

1. High numerical aperture (NA) lens. NA = 0.5 is a recommended minimum for a ×40 lens.

2.2.1. Live Visualization of Fluorescent Reporters

1. Culture, recording, or imaging medium without phenol red or other dyes.
2. Appropriate microscope filters for single-, double-, or triple-labeling (Omega Optical, Brattleboro, VT; Chroma Technology, Brattleboro, VT) (*see* **Note 5**).

2.2.2. Fixed Specimens

1. 4% paraformaldehyde solution (prepared fresh).
2. Phosphate-buffered saline (PBS) for washing and storage.
3. Appropriate reagents for secondary labeling, as desired: 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) solution, antibodies to gene of interest, and so on.

3. Methods

3.1. Viral Harvest and Concentration

1. Harvest the medium from the packaging cells and subject to a preliminary round of centrifugation at ~300 × g for 10 min at 4°C. This is necessary to pellet large debris from the 293T cells; such debris can be toxic to cultured neurons.
2. Pass through a 0.45-μm filter (*see* **Note 6**).

3.1.1. Ultrafiltration

1. Transfer the supernatant into the unit and subject to two rounds of centrifugation at the highest recommended speed. The resulting virus is concentrated approx 30-fold and may be frozen in 1.0- to 1.5-mL cryogenic vials at –80°C until needed.

3.1.2. Ultracentrifugation

1. Centrifuge the supernatant for 1.5 h at ~30,000 × g.
2. Transfer the centrifuge tubes into an appropriate containment hood. Invert the tubes and allow all traces of the packaging cell culture medium to drain into a waste container. The pellet will not be visible (*see* **Note 7**). Vacuum suction may be used to remove traces of medium (being careful not to touch the bottom of the tube), or the centrifuge tubes may be inverted on a sterile tissue for 5–10 min. It is important to remove all traces of medium as serum is harmful to the viruses ***(24)***.
3. After the medium is drained away, pipet 20–50 μL (for a 12-mL centrifuge tube) or 60–200 μL (for a 30-mL centrifuge tube) TBS into the bottom of the tube. Cover the tubes with Parafilm and allow to sit 2 h to overnight (if left overnight, put at 4°C). Resuspend vigorously with a micropipet, dispense into 5- to 10-μL aliquots, and store at –80°C (*see* **Notes 8** and **9**).

3.2. Culture System and Age In Vitro

1. Choose the culture system and grow the neurons to the desired age.
2. For Banker cultures, remove the glial coverslip.
3. Remove virus from –80°C storage and thaw at room temperature or at 37°C (*see* **Note 10**).
4. For any culture, add 2–5 μL of ultracentrifuged virus to the culture medium (1–2 μL for multielectrode arrays). If using filtered virus, add 100–500 μL after removing an equal amount of medium from the dish (*see* **Note 11**).
5. Washing out the virus is unnecessary except in the case of multielectrode arrays, in which it appears to help survival. Do not wash before approx 12–24 h have elapsed and never expose neurons to an air-water interface.
6. We recommend parallel production of 6 to 12 identical "sister" cultures if data on infection time courses are desired.

3.3. Reporter Genes

Do not assay before 36–48 h (*see* **Note 12**).

3.3.1. Live Visualization of Fluorescent Reporters

1. Rinse the culture dish in medium without phenol red (which will cause background fluorescence).
2. Visualize using the appropriate magnification and filter sets. Very low levels of GFP expression may require confocal imaging or staining with anti-GFP antibody (*see* **Subheading 3.3.2.**).
3. Culture dishes should be discarded after opening, as contamination is inevitable.
4. If no GFP is seen, examine a sister culture after an additional 24–48 h.

3.3.2. Fixed Specimens

1. Fix for 10–30 min in 4% paraformaldehyde.
2. Wash thoroughly with PBS.
3. Develop lacZ according to standard protocols. LacZ-stained cultures may be visualized days to weeks after staining if they are kept moist and protected from physical damage (shaking, splashing).
4. For fixed studies and immunolabeling, visible GFP may not remain after fixation and will certainly be absent after permeabilization unless an anchored GFP is used. Both nuclear GFP **(Fig. 5A**,**B)** and Actin-GFP **(Fig. 5C**,**D)** work well for this purpose; the location of the targeting can be chosen to correspond with the immunolabel or other secondary fluorophore.
5. For visualization of dendritic spines, ordinary cytosolic GFP does not yield good labeling until at least 5–7 d postinfection **(Fig. 5E)**. GFPs targeted to spines may be created using, for example, the 5′ untranslated region of the CaMK II promoter ***(25)***; these are not yet available commercially.

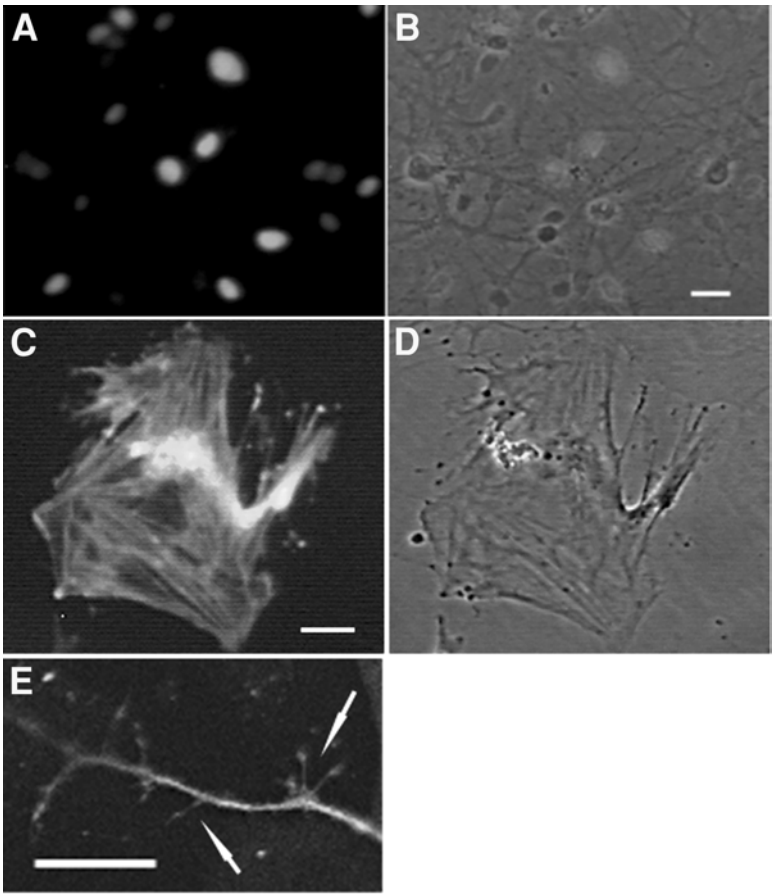

Fig. 5. Appearance of targeted fluorescent proteins in lentivirally infected hippocampal neurons. Scale bar = 20 μm. **(A**,**B)** Nuclear targeting. In the fluorescent image (A), it is difficult to tell neurons from glia. An overlay (B) of fluorescent and phase-contrast images reveals those nuclei that belong to neurons (arrows) and those that are part of glial cells (whose cell bodies are nearly invisible under phase contrast). Glial cells express GFP more strongly and have larger nuclei than neurons. **(C**,**D)** Actin-GFP fusion constructs label the cell's cytoskeleton, as seen in this large astrocyte. **(E)** Nontargeted GFP labels dendritic spines (arrows) after extended time periods. 21 dic, 10 dpi.

4. Notes

1. A word of caution is in order for IRES/CITE constructs. A set of Clontech (Palo Alto, CA) vectors ("p-IRES") contained a partially disabled IRES sequence, in which expression of the second gene was greatly reduced. It was not possible to visualize GFP using these vectors. The most recent vectors ("p-IRES2") contain

a different IRES and are reported to work for direct visualization. We have used pCITE vectors (Novagen, Madison, WI) with good results.

2. IRESes have been identified in neuronal genes encoding dendritically-localized proteins, e.g., CaMK II, MAP2, and others. Bicistronic constructs using these IRESes have been shown to allow cap-independent, dendritically localized transcription in calcium phosphate-transfected neurons ***(26)***. This holds promise for construction of novel bicistronic lentiviruses, but to our knowledge, such vectors have not yet been attempted.
3. Alternatively, neurons may be grown on coverslips inverted over a dish of astrocytes. An excellent reference for preparation of all types of hippocampal culture is *Culturing Nerve Cells*, Gary Banker and Kimberly Goslin, eds., the MIT Press, Second Ed. 1998.
4. Use of 15-mL filter units achieves a practical virus concentration for cultured cell use. Attempts to concentrated further using smaller units (1–2 mL units for concentration to μL volumes) lead to destruction of viral particles and loss of titer.
5. BFP, GFP, and red fluorescent protein are all readily distinguished by eye. CFP and YFP appear very similar to GFP, and are more useful for multilabeling experiments in which the images are postprocessed using a deconvolution algorithm ***(27)***.
6. Before disposing of the packaging cells, they may be tested for expression of a reporter gene if there is any doubt about the vector's function or the transfection efficiency. For GFP-containing vectors, GFP levels are extreme: the fluorescence is readily seen even over bright-field illumination.
7. If anything is visible in the bottom of the tube, it is traces of cellular debris from the first step and will be very viscous. It indicates insufficient centrifugation and/or filtration of the original supernatant; this debris causes viral particles to clump, reducing infection efficiency, and may be toxic to neurons.
8. While there have been conflicting reports about the effects of freeze-thaw on viral titers, it is generally accepted that vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped particles should not be subjected to freeze-thaw cycles ***(24)***.
9. Effort should be made to keep the viral free of bacterial and fungal contamination throughout these steps; however, we have not found that any special sterilization procedures are necessary. The rotor bucket mouths should be wiped with 70% ethanol, and a fresh box of centrifuge tubes should be opened and then stored within the hood or in a similar sterile environment. Although the tubes are not advertised as sterile, we have experienced no contamination after following these handling guidelines.
10. Nondividing cells often maintain low cytoplasmic concentrations of free nucleotides, which are required for reverse transcription of viral RNA into provirus DNA. For this reason, reverse transcription has been suggested to be the rate-limiting step in retroviral infection of quiescent cells, and early papers recommended that viral stocks be preincubated with deoxynucleotide 5′-triphosphates (dNTPs) to encourage reverse transcription inside the viral particle before the particles are applied to the cells ***(28)***. In a series of experiments, we found

no effect on kinetics or infection efficiency from this practice in vitro, and no longer use it.

11. Many compounds are available to encourage viral binding to cultured cells. We did not find appreciable benefits of any of them, including the recombinant fibronectin RetroNectin (TaKaRa Biomedicals, Japan). Others, such as wheat germ agglutinin (WGA), are toxic to neurons. If binding appears inefficient, lowering the infection volume is the most effective remedy.
12. There is an absolute limit on the speed of lentiviral transduction. Virus must encounter the cell, enter the cytoplasm, reverse transcribe, interact with cytoplasmic chaperones and viral enzymes to create a high-molecular weight preincubation complex, and finally this complex must target the nucleus, cross the nuclear membrane, and integrate into the genome ***(29)***. Hence, while GFP may begin to appear in dividing cells after 24 h or less, examining differentiated neurons with even the most sensitive methods is fruitless before approx 2 d have elapsed.

References

1. Wiesenhofer, B. and Humpel, C. (2000) Lipid-mediated gene transfer into primary neurons using FuGene: comparison to C6 glioma cells and primary glia. *Exp. Neurol.* **164,** 38–44.
2. Sato, H., Hattori, S., Kawamoto, S., et al. (2000) In vivo gene gun-mediated DNA delivery into rodent brain tissue. *Biochem. Biophys. Res. Commun.* **270,** 163–170.
3. Aakalu, G., Smith, W. B., Nguyen, N., Jiang, C., and Schuman, E. M. (2001) Dynamic visualization of local protein synthesis in hippocampal neurons. *Neuron* **30,** 489–502.
4. Xu, R., Janson, C. G., Mastakov, M., et al. (2001) Quantitative comparison of expression with adeno-associated virus (AAV- 2) brain-specific gene cassettes. *Gene Ther.* **8,** 1323–1332.
5. Lundstrom, K., Rotmann, D., Hermann, D., Schneider, E. M., and Ehrengruber, M. U. (2001) Novel mutant Semliki Forest virus vectors: gene expression and localization studies in neuronal cells. *Histochem. Cell Biol.* **115,** 83–91.
6. Ehrengruber, M. U., Hennou, S., Bueler, H., Naim, H. Y., Deglon, N., and Lundstrom, K. (2001) Gene transfer into neurons from hippocampal slices: comparison of recombinant Semliki Forest Virus, adenovirus, adeno-associated virus, lentivirus, and measles virus. *Mol. Cell Neurosci.* **17,** 855–871.
7. D'Apuzzo, M., Mandolesi, G., Reis, G., and Schuman, E. M. (2001) Abundant GFP expression and LTP in hippocampal acute slices by in vivo injection of sindbis virus. *J. Neurophysiol.* **86,** 1037–1042.
8. Nadeau, H., McKinney, S., Anderson, D. J., and Lester, H. A. (2000) ROMK1 (Kir1.1) causes apoptosis and chronic silencing of hippocampal neurons. *J. Neurophysiol.* **84,** 1062–1075.
9. Blomer, U., Naldini, L., Kafri, T., Trono, D., Verma, I. M., and Gage, F. H. (1997) Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J. Virol.* **71,** 6641–6649.

10. Gasser, U. E. and Hatten, M. E. (1990) Neuron-glia interactions of rat hippocampal cells in vitro: glial-guided neuronal migration and neuronal regulation of glial differentiation. *J. Neurosci.* **10,** 1276–1285.
11. Berger, T. and Frotscher, M. (1994) Distribution and morphological characteristics of oligodendrocytes in the rat hippocampus in situ and in vitro: an immunocytochemical study with the monoclonal Rip antibody. *J. Neurocytol.* **23,** 61–74.
12. Liu, X., Constantinescu, S. N., Sun, Y., et al. (2000) Generation of mammalian cells stably expressing multiple genes at predetermined levels. *Anal. Biochem.* **280,** 20–28.
13. Zufferey, R., Donello, J. E., Trono, D., and Hope, T. J. (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73,** 2886–2892.
13a. Nadeau, H. and Lester, H. A. (2002) NRSF causes cAMP-sensitive suppression of sodium current in culture dhippocampal neurons. *J Neurophysiol.* **88,** 409–421.
14. Liu, H. S., Jan, M. S., Chou, C. K., Chen, P. H., and Ke, N. J. (1999) Is green fluorescent protein toxic to the living cells? *Biochem. Biophys. Res. Commun.* **260,** 712–717.
15. Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline- responsive promoters. *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
16. Kafri, T., van Praag, H., Gage, F. H., and Verma, I. M. (2000) Lentiviral vectors: regulated gene expression. *Mol. Ther.* **1,** 516–521.
17. Seo, M. L., Yamatodani, A., Mizutani, A., and Kiyono, S. (1993) Short-term, preweaning treatment with tetracycline affects physical development and behavior in rats. *Neurotoxicology* **14,** 65–75.
18. Navarro, V., Millecamps, S., Geoffroy, M. C., et al. (1999) Efficient gene transfer and long-term expression in neurons using a recombinant adenovirus with a neuron-specific promoter. *Gene Ther.* **6,** 1884–1892.
19. Wang, S., Bui, V., Hughes, J. A., King, M. A., and Meyer, E. M. (2000) Adeno-associated virus mediated gene transfer into primary rat brain neuronal and glial cultures: enhancement with the pH-sensitive surfactant dodecyl 2-(1′-imidazolyl) propionate. *Neurochem. Int.* **37,** 1–6.
20. Andersen, J. K., Frim, D. M., Isacson, O., and Breakefield, X. O. (1993) Herpesvirus-mediated gene delivery into the rat brain: specificity and efficiency of the neuron-specific enolase promoter. *Cell Mol. Neurobiol.* **13,** 503–515.
21. Lessmann, V. and Heumann, R. (1997) Cyclic AMP endogenously enhances synaptic strength of developing glutamatergic synapses in serum-free microcultures of rat hippocampal neurons. *Brain Res.* **763,** 111–122.
22. Liu, Q. Y., Schaffner, A. E., Chang, Y. H., Vaszil, K., and Barker, J. L. (1997) Astrocytes regulate amino acid receptor current densities in embryonic rat hippocampal neurons. *J. Neurobiol.* **33,** 848–864.
23. Potter, S. M. and DeMarse, T. B. (2001) A new approach to neural cell culture for long-term studies. *J. Neurosci. Methods* **110,** 17–24.

24. Higashikawa, F. and Chang, L. (2001) Kinetic analyses of stability of simple and complex retroviral vectors. *Virology* **280,** 124–131.
25. Mayford, M., Baranes, D., Podsypanina, K., and Kandel, E. R. (1996) The 3′-untranslated region of CaMKII alpha is a cis-acting signal for the localization and translation of mRNA in dendrites. *Proc. Natl. Acad. Sci. USA* **93,** 13,250–13,255.
26. Pinkstaff, J. K., Chappell, S. A., Mauro, V. P., Edelman, G. M., and Krushel, L. A. (2001) Internal initiation of translation of five dendritically localized neuronal mRNAs. *Proc. Natl. Acad. Sci. USA* **98,** 2770–2775.
27. Lansford, R., Bearman, G., and Fraser, S. E. (2001) Resolution of multiple green fluorescent protein color variants and dyes using two-photon microscopy and imaging spectroscopy. *J. Biomed. Opt.* **6,** 311–318.
28. Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. USA* **93,** 11,382–11,388.
29. Bukrinsky, M. I., Sharova, N., Dempsey, M. P., Stanwick, T. L., Bukrinskaya, A. G., Haggerty, S., and Stevenson, M. (1992) Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc. Natl. Acad. Sci. USA* **89,** 6580–6584.

13

Dorsal Root Ganglia Sensory Neurons

Jane Fleming, Samantha L. Ginn, and Ian E. Alexander

1. Introduction

The ability of lentivirus vectors to stably transduce postmitotic cells by genomic integration and early evidence of a capacity for retrograde axoplasmic transport, depending on the envelope pseudotype, underpin the immense potential of this vector system for neuronal transduction ***(1–3)***. The bulk of studies performed to date have focused on central nervous system (CNS) neurons despite the relative merits of peripheral nervous system (PNS) neurons as targets for gene transfer ***(4–6)***. The neural circuitry of the PNS is relatively simple, accessible, and anatomically discrete. Dorsal root ganglion (DRG) sensory neurons exemplify these properties, are readily cultured ex vivo after dissociation or as intact DRG explants, and as a consequence, have been extensively exploited as a model system by neurobiologists ***(7)***. The demonstrated ability of human immunodeficiency virus type 1 (HIV-1)-derived lentivirus vectors to stably transduce DRG sensory neurons ***(8)*** extends the utility of this model system for both basic science investigations of neuronal structure and function and for the development and evaluation of gene therapy strategies targeting PNS neuropathology.

High-efficiency transduction of sensory neurons can be achieved in dissociated cultures at multiplicities of infection (MOI) as low as 10 transducing units per cell using vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped HIV-derived lentivirus vectors. Direct microinjection of intact explants with as little as 1–2 μL of high-titer vector is also capable of achieving widespread neuronal transduction within the interstitium of an explant. Importantly, current generation vectors incorporating the central polypurine tract (cPPT), a posttranscriptional regulatory element (PRE), and a self-inactivating (SIN)

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deletion in the U3 region of the long terminal repeat (LTR) perform similarly in DRG cultures of human and murine origin. Each of the three major cell types present, sensory neurons, Schwann cells, and fibroblast-like cells, are efficiently and stably transduced. Early generation lentivirus vectors exhibited a propensity for neuron-specific transduction in murine, but not human, cultures (Ginn et al., manuscript submitted). Enhanced green fluorescent protein (EGFP) is a particularly useful reporter in this system, allowing gene expression to be monitored in individual cultures for several weeks, during which time EGFP is distributed and readily visible within axonal projections.

This chapter provides the essential methodologies and advice for successful experimental use of lentivirus vectors as tools for gene transfer to cultured DRG sensory neurons. These include (1) the preparation and characterization of vector stocks, (2) the preparation and maintenance of DRG cultures with assiduous attention to sterility, and (3) the use of adequate multiplicities of infection to achieve efficient, stable, and readily detectable transgene expression.

2. Materials

All reagents should be of tissue culture grade. Cell and explant cultures are maintained at 37°C in a humidified 5% CO_2-air atmosphere. All reagents are prepared with sterile distilled water and stored at room temperature unless stated otherwise.

2.1. Preparation of Lentivirus Stocks, Concentration, and Characterization

1. Human embryonic kidney (HEK) 293 cells ***(9)***.
2. Growth medium for HEK 293 cells: Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Commonwealth Serum Laboratories, Parkeville, Melbourne) and 2 m*M* glutamine. Store at 4°C.
3. T75 tissue culture flasks with vented caps.
4. 2X N-2-hydroxy ethylpiperazine-N-2-ethanesulfonic acid (HEPES)-buffered saline (HBS): 280 m*M* NaCl, 50 m*M* HEPES. Adjust to exactly pH 7.1 and autoclave.
5. 150 m*M* Na_2HPO_4. Adjust to exactly pH 7.1 with 1 *N* NaOH and autoclave.
6. 10% TE: 1 m*M* Tris-HCl, 0.1 m*M* ethylenediaminetetraacetic acid (EDTA). Adjust to pH 8.0 and autoclave.
7. 2 *M* $CaCl_2$. Sterilize by autoclaving.
8. 14-mL polypropylene round-bottom tubes (BD Falcon, Franklin Lakes, NJ).
9. Affinity purified plasmid DNA (Qiagen Plasmid Maxi Kit, Hildan, Germany).
10. 10-mL syringes.
11. Plastic mixing cannulas.

12. 0.45-μm syringe filters, cellulose acetate membrane.
13. Vivaspin 20-mL concentrator, 100,000 molecular weight cut-off (MWCO), polyethersulfone (PES) membrane (Sartorius, AG, Guettingen, Germany).
14. 24-well tissue culture plate.
15. Polybrene (Sigma-Aldrich, St. Louis, MO). Prepare a 4 mg/mL working stock and sterilise by filtration. Aliquot and store at –20°C.
16. Inverted fluorescent microscope with appropriate excitation and emission filter of 450 to 490 nm wavelength to detect EGFP.
17. CEM cells, a human T lymphoblastoid cell line ***(10)***.
18. Growth medium for CEM cells; RPMI 1640 medium (GIBCO-BRL) supplemented with 10% bovine calf serum (CS; Starrate, Bethungra, NSW, Australia) and 2 m*M* glutamine. Store at 4°C.
19. HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) kit (NEN Life Science Products, Boston, MA).

2.2. Preparation of DRG Cultures

2.2.1. Preparation of Rat Tail Collagen (RTC) and Coating of 24-Well Plates

1. Source of young adult rat tails (e.g., Wistar) from which RTC is to be extracted.
2. Hibiclens antiseptic skin cleanser (ICI, Melbourne, Victoria, Australia).
3. 80% ethanol.
4. Haemostats and forceps.
5. 0.1% acetic acid. Store at 4°C.
6. Slide-A-Lyser dialysis cassettes, 10,000 MWCO, 3 to 15 mL sample volume (Pierce, Rockford, IL).
7. Ammonium hydroxide (A.C.S reagent) (Sigma-Aldrich). Store at 4°C.

2.2.2. Dissection of Newborn Mouse DRG

1. Source of newborn mice (e.g., Swiss-White) from which DRG are to be extracted.
2. 100% ethanol.
3. Watchmakers forceps, Dumostar, Dumont #5 (World Precision Instruments, Sarasota, FL).
4. Vannas dissecting scissors OC498R (Aesculap, Tuttelingen, Germany).
5. Nerve growth factor 7S (NGF; Becton Dickinson, Franklin Lakes, NJ). Prepare a 50 mg/mL working stock, aliquot, and store at –20°C.
6. DRG medium for mice: Eagle's minimal essential medium (EMEM; GIBCO-BRL), 10% Horse serum (Sigma-Aldrich), 10% CS, 2 m*M* glutamine. Store at 4°C.

2.2.3. Dissection of Fetal Human and Fetal Sheep DRG

1. Source of tissue from which DRG are to be extracted.
2. Hibernate E (GIBCO-BRL). Store at 4°C.
3. Delicate dissecting scissors BC100 (Aesculap).

4. Micro forceps BD331 (Aesculap).
5. NGF. Prepare a 50 mg/mL working stock, aliquot, and store at –20°C.
6. DRG medium for sheep and human: DMEM, 15% FBS, 1X ITS-1 liquid media supplement with insulin, transferrin, and selenium (Sigma-Aldrich), 5.1 g/L D-glucose, 2 m*M* glutamine. Store at 4°C.

2.2.4. Dissociation of Mouse DRG

1. 0.25% (w/v) Trypsin (Boehringer Mannheim, Indianapolis, IN) in EMEM. Store at –20°C in 5 mL aliquots.
2. Serum-free EMEM. Store at 4°C.
3. 18-gauge needles.
4. 15-mL polypropylene tube (BD Falcon).
5. 24-well tissue culture plates.

2.2.5. Culture of Dissociated DRG and Explants

1. 10 μ*M* cytosine arabinoside (Sigma-Aldrich). Store at –20°C.
2. Lab-Tek® 2-well glass chamber slides (Nalge-Nunc, Naperville, IL).

2.3. Transduction of Sensory Neurons and Analysis of Transgene Expression

2.3.1. Transduction of Sensory Neurons

1. Characterized lentivirus vector stocks.
2. DRG cultures.
3. Inverted fluorescent microscope with appropriate excitation and emission filter of 450–490 nm wavelength to detect EGFP.

2.3.2. Immunohistochemistry—Dissociated Cells

1. 4% Paraformaldehyde. Make up fresh and cool to 4°C.
2. Phosphate-buffered saline (PBS) (+/+, with Ca^{2+} and Mg^{2+}).
3. Methanol. Store at –20°C.
4. PBS (–/–, without Ca^{2+} and Mg^{2+}).
5. Blocking solution: 0.1% sodium azide, 15% goat serum (GIBCO-BRL), 10% CS. Make up fresh.
6. NeuN nuclear neuron-specific mouse monoclonal antibody (Chemicon, El Segundo, CA). Store at 4°C.
7. Goat antimouse secondary antibody IgG conjugated to rhodamine red (Jackson Immunoresearch, West Grove, PA). Store at –20°C in 100 μL aliquots.
8. 4,6 diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Prepare a 0.1 μg/mL stock in PBS (–/–). Store at 4°C.
9. Inverted fluorescent microscope with appropriate excitation and emission filters of 340–425 nm wavelength to detect DAPI, 450–490 nm wavelength to detect EGFP, and 515–560 nm wavelength to detect rhodamine red.

2.3.3. Immunohistochemistry—Explants

1. Sucrose solutions 5, 10, 15, 20, 25, and 30% (w/v). Store at 4°C.
2. Tissue-Tek Cryomolds (Miles, Elkhart, IN).
3. Tissue-Tek OCT (Miles).
4. Isopentane.
5. Poly-L-lysine-coated slides (HD Scientific Supplies, Kings Park, NSW, Australia).
6. Immunomount (Shandon).
7. Liquid-repellant slider marker PAP pen (Daido Sangyo, Tokyo, Japan).

3. Methods

3.1. Preparation of Lentivirus Stocks, Concentration, and Characterization

For more detailed protocols refer to Chapters 3 through 6. Methods are adapted from the protocol of Naldini and co-workers ***(11)***. The plasmids described herein for lentivirus vector production, are those used by the authors (*see* **Note 1**). If other plasmid reagents are used, the relative individual amounts should be optimised empirically.

1. Depending on amount of vector required, seed an appropriate number of T75 flasks with HEK 293 cells in growth medium so that they are approximately 90% confluent on the day of transfection (*see* **Note 2**).
2. Day 1: Replace growth medium (6 mL) on the HEK 293 cell monolayer.
3. Prepare transfection mixes (per T75 flask) in 14-mL polypropylene round-bottom tubes as follows: Tube A, 5 µL 150 m*M* Na_2HPO_4 and 500 µL 2X HBS; Tube B, 20 µg of the EGFP-encoding vector plasmid pHR′CMV-EGFP, 15 µg of a packaging plasmid such as pCMVΔR8.91, 5 µg of the VSV-G envelope-encoding plasmid pMD.G, 64 µL 2 *M* $CaCl_2$ (add last), and 10% TE to 500 µL final volume (*see* **Note 3**).
4. Transfer the contents of Tube B to Tube A dropwise, mixing the tube intermittently. Incubate at room temperature for 20 min. The precipitate should make the solution appear slightly opalescent.
5. Add the precipitate (1 mL) to the cellular monolayer and incubate overnight (*see* **Note 4**).
6. Day 2: Carefully replace medium (10 mL) on the cellular monolayer.
7. Days 3 through 5: Collect the vector supernatant using a 10-mL syringe and mixing cannula, pass through a 0.45-µm syringe filter, and store at –80°C (*see* **Note 5**).
8. Prepare concentrated lentivirus vector stocks by centrifugation using a Vivaspin 20-mL concentrator. Place 20 mL of vector supernatant in a Vivaspin concentrator and spin at 2000*g* at room temperature for 90 min or until the desired concentration is achieved (approx 100-fold). Store concentrated vector stock in 20–50-µL aliquots at –80°C.

9. Assign lentivirus titers on HEK 293 cells. Plate cells at a seeding density of 5×10^4 cells/well in a 24-well tissue culture plate. The following day add serial dilutions of vector supernatant and concentrated stock to duplicate wells in the presence of Polybrene (8 μg/mL of culture medium). Calculate lentivirus titer by counting transduction events 48 h after vector exposure (*see* **Note 6**).
10. Test lentivirus vector stocks for replication-competent retrovirus (RCR) by inoculating HEK 293 and CEM cell cultures with 1 mL of vector supernatant. Passage cells twice weekly for at least 4 wk. Harvest supernatant, filter through a 0.45-μm syringe filter and store at –80°C. Perform HIV-1 p24 ELISA on the collected supernatant as per the manufacturer's instructions (*see* **Note 7**).

3.2. Preparation of DRG Cultures

3.2.1. Preparation of RTC and Coating of 24-Well Plates (12)

1. Remove tails from two young adult rats, wash with Hibiclens, and collect into one 50-mL polypropylene tube. Rinse tails in 80% ethanol and place on blotting paper in a sterile petri dish at –20°C overnight. Using aseptic technique in a laminar flow hood, wash each tail in 80% ethanol for 20 min and then air-dry.
2. Grasp tail 1 cm from the tip with a pair of haemostats and at 1.5 cm intervals from the top score the skin with a pair of scissors and then break bones using strong forceps/haemostats. Pull bones apart exposing the tendons and cut into a preweighed petri dish of sterile water. Grasp the tail at the top and repeat again until all tendons are extracted.
3. Transfer tendons to 0.1% acetic acid (150 mL/g of tendon) and stir for 5 d in a cold room at 4°C. During this time, the tendons release soluble collagen. Centrifuge collagen (10,000*g*, 30 min) to remove undissolved tendons, transfer supernatant into three 15-mL Slide-A-Lyser cassettes and dialyze against sterile distilled water (50 vols) overnight. Pipet RTC into sterile eppendorfs and store at –80°C until use.
4. In tissue culture hood, coat a 24-well plate with two drops of RTC. Spread solution onto the surface of each well using a plastic pipet. Invert plate over a tissue soaked in ammonium hydroxide for 2 min. Air-dry plate in a laminar flow hood and if necessary store for up to 1 wk at 4°C.

3.2.2. Dissection of Newborn Mouse DRG (13,14)

1. Take four newborn mice (0 to 3 d) and decapitate.
2. In a laminar flow hood, wash the dorsal spinal region of the mouse with Hibiclens and 100% ethanol. Place the body in a 6-cm petri dish and proceed with dissection using aseptic technique.
3. Dissect out vertebral column close to the ribs, leaving approx 1–2 mm of ribs attached. Transfer vertebral column to a sterile petri dish, place in PBS (5 mL), and remove extraneous tissue using watchmakers forceps.
4. Using a dissecting microscope, insert dissecting scissors into the rostral end of the vertebral column and cut open centrally along the ventral axis two to three

vertebrae at a time. Turn the vertebral column over and cut along the dorsal axis and separate the two halves.

5. Using two pairs of watchmakers forceps carefully remove the spinal cord, checking for the occasional attached DRG. Locate the remaining DRG between the vertebrae and the ribs, and using one pair of forceps to anchor the vertebral column to the dish, use the second pair to pluck the DRG from between the vertebrae. Remove extraneous membrane, bone, or excess nerve root and collect DRG to one side of the petri dish in PBS (*see* **Fig. 1A** and **Note 8**). When all the DRG have been removed from one mouse, transfer the DRG to a sterile petri dish containing DRG medium for mice and 50 ng/mL fresh NGF (*see* **Notes 9** and **10**).

3.2.3. Dissection of Fetal Human and Fetal Sheep DRG **(15)**

1. Collect tissue into ice cold Hibernate E medium (*see* **Note 11**). In a laminar flow hood, take a piece of vertebral column and place in a sterile petri dish containing 100% ethanol for 30 s. Transfer the vertebral column into another petri dish containing sterile PBS for 30 s. Repeat ethanol and PBS wash (*see* **Note 12**).
2. Place vertebral column into a sterile petri dish containing approx 5 mL PBS. Using small scissors cut the vertebral column open along the ventral axis. Then cut the vertebral column along the dorsal axis and separate the halves.
3. Using two pairs of fine forceps carefully remove the spinal cord as above checking for attached DRG. Identify detached DRG between the vertebrae and, using a fine pair of forceps, pluck from the vertebral column (*see* **Note 13**).
4. Remove extraneous tissue including the capsule from the DRG, which can be peeled away using two pairs of fine forceps. Collect DRG in to a corner of the petri dish in PBS (*see* **Note 14**), and then transfer to a sterile petri dish containing DRG medium for sheep and human and 50 ng/mL fresh NGF (*see* **Notes 9**, **10**, and **15**).

3.2.4. Dissociation of Mouse DRG

1. In a tissue culture hood, transfer the DRG from four newborn mice to one 15-mL polypropylene tube. Centrifuge gently (100*g*, 3 min), remove medium, and replace with 2 mL 0.25% trypsin in serum-free EMEM medium.
2. Place the DRG in trypsin in a shaking incubator (~200 rpm) at 37°C for 30–45 min. Centrifuge (100*g*, 3 min), remove trypsin solution, and resuspend in 1 mL fresh medium and transfer to a petri dish.
3. Dissociate cells using 10 passes through an 18-gauge needle, transfer cells to a 15-mL polypropylene tube, rinse petri dish and syringe with 1 mL fresh medium, combine and centrifuge (100*g*, 3 min). Resuspend cell pellet in 1 mL medium containing NGF.

3.2.5. Dissociation of Human and Sheep DRG

Dissociate human and sheep DRG as above, but mince tissue finely with a scalpel blade before passing cells through an 18-gauge needle.

A
B
C
D
E
F

3.2.6. Culture of Dissociated DRG and Explants

1. Plate dissociated cells into four wells of a 24-well plate and maintain in the appropriate DRG growth medium containing 50 ng/mL fresh NGF (*see* **Note 9**). Change medium every 3–4 d. Add 50 ng/mL fresh NGF at each medium change. Cultures are routinely maintained for 4 wk. If required, fibroblast and Schwann cell numbers can be significantly reduced by including cytosine arabinoside in the culture medium from d 7 to d 10 (*see* **Note 16**).
2. Culture explants in 2-well glass chamber slides (~10/well) as above (*see* **Note 17**).

3.3. Transduction of Sensory Neurons and Analysis of Transgene Expression

3.3.1. Transduction of Sensory Neurons

1. Add lentivirus vector stocks directly to the culture medium of dissociated DRG cultures, in the absence of polybrene at estimated MOIs of between 10 and 20. Monitor transgene expression over time by fluorescence microscopy (*see* **Fig. 1B**,**C** and **Notes 18–20**).
2. Add lentivirus vector stocks directly to the culture medium after DRG explants are well established (*see* **Fig. 1D**) or deliver by microinjection of 1–2 μL of vector stock after 4 to 5 d (*see* **Note 21**). Monitor transgene expression over time by fluorescence microscopy (*see* **Note 22**).

3.3.2. Immunohistochemistry—Dissociated Cells

To establish the number of neurons transduced, DRG cultures or explants can be immunohistochemically stained with the neuron-specific monoclonal antibody NeuN.

1. Fix and permeabilize dissociated cells in ice cold 4% paraformaldehyde for 30 min and wash three times in PBS (+/+) for 5–10 min (*see* **Note 23**).
2. Further fix and permeabilize in ice cold methanol for 30 min and wash three times in PBS (–/–) containing 2% CS for 5–10 min.
3. Incubate in blocking solution overnight at 4°C.

Fig. 1. *(see facing page)* **(A)** Microphotograph of a newborn mouse DRG (size bar indicates 100 μm). **(B**,**C)** EGFP expression in dissociated murine DRG cultures after exposure to HR′CMV-EGFP lentivirus vector at an estimated MOI of 100. Panels B and C show representative fields at 3 and 7 d, respectively, after vector exposure (×200 magnification). **(D)** EGFP expression in newborn mouse DRG explant culture 3 wk after exposure to 2×10^7 transducing units of RRLsin18-cPPT-CMV-EGFP-WPRE ***(19)*** (×100 magnification). **(E**,**F)** EGFP expression in dissociated human DRG cultures after exposure to HR′CMV-EGFP at an estimated MOI of 20. Panels E and F show EGFP and NeuN images, respectively, of the same field 10 d after vector exposure (×200 magnification).

4. Add neuron-specific primary antibody NeuN diluted (1:250) in PBS (–/–) and incubate for 1 h at room temperature. Wash three times in PBS (–/–) containing 2% CS for 5–10 min.
5. Add secondary antibody Goat antimouse IgG rhodamine red diluted (1:250) in PBS (–/–) and incubate for 1 h at room temperature in the dark. Wash three times in PBS (–/–) for 5–10 min in the dark. DAPI counter stain at this point if required. Add DAPI for 5 min at room temperature in the dark. Wash cells in PBS (–/–) for 5 min (*see* **Note 24**).
6. Leave a film of PBS covering cells and visualise fluorescence using an inverted fluorescent microscope (*see* **Fig. 1E**,**F** and **Note 25**).

3.3.3. Immunohistochemistry—Explants (Frozen Sections)

1. Pluck DRG explants from tissue culture plates using fine forceps and place in fresh ice cold 4% paraformaldehyde in a Petri dish for 30 s to 2 min. Remove DRG from paraformaldehyde and place in a graded series of ice cold sucrose solutions (5–30%) for 12–16 h each at 4°C.
2. Place DRG in a small foil mould or labelled cryomold containing a bed of optimal cutting temperature (OCT) compound. Fill the mould with OCT and place in a plastic beaker of isopentane precooled in a container of liquid nitrogen. When the OCT becomes white in color (10–30 s), remove mould to liquid nitrogen for 30 min. Transfer mould to dry ice for a few minutes, remove block from the mould, and store at –80°C, labelled and wrapped in foil.
3. Cut 5-10 μm sections from DRG-containing blocks on a cryostat at –20°C and collect sections onto poly-L-lysine-coated slides. Antibody stain slides as above. Slides can be divided using a PAP pen. Mount slides using immunomount.

4. Notes

1. Sensory neurons have been successfully transduced with HR′CMV-EGFP ***(8)*** lentivirus stocks packaged with the second-generation constructs pCMVΔR8.2 ***(11)***, pCMVΔRnr ***(16)***, and pCMVΔR8.91 ***(6)***. Neuronal transduction has also been observed with the transfer vectors RRL-CMV-EGFP-SIN, RRL-cPPT-CMV-EGFP-SIN, RRL-CMV-EGFP-PRE-SIN, and RRL-cPPT-CMV-EGFP-PRE-SIN ***(17)***. All lentivirus vectors described herein contain an EGFP expression cassette under the control of a human cytomegalovirus (CMV) immediate early promoter. The functional utility of alternate transgenes and promoters should be established empirically.
2. Using the protocol described, the yield of functional virions ranges from 2×10^6 to 2×10^7 transducing units per T75 flask, unconcentrated.
3. If self-inactivating lentivirus stocks are to be prepared, 13 μg of the vector plasmid are used. Third-generation lentivirus stocks are produced by using 8.5 μg of pMDLg/pRRE, 6.5 μg of pRSV-Rev ***(18)***, and 5 μg of pMD.G.
4. Transfection efficiencies of ≥90% are readily achieved in HEK 293 cells.
5. VSV-G-induced syncytia formation should be observed in the HEK 293 cellular monolayer by d 5.

6. Forty-eight hours following exposure, HEK 293 cells will have undergone cell division. As a result, clusters of EGFP positive cells should be scored as a single transduction event.
7. Following at least 4 wk of serial passaging, samples should have absorbance values below the manufacturer prescribed cut-off factor. This indicates the absence of RCR in the sample of vector stock tested.
8. Approximately 30–40 DRG are routinely collected from each newborn mouse.
9. Nerve growth factor preparations from different suppliers may vary in activity. The ability of NGF to differentiate PC12 cells (PC12 is a rat pheochromocytoma cell line: American Type Culture Collection) can be used to assess NGF activity.
10. Neurons tolerate antibiotics and fungicides poorly, therefore, prepare cultures aseptically.
11. Neuronal tissue collected from fetal human and fetal sheep can be maintained in Hibernate E medium on ice up to 24 h before dissection without any obvious loss of viability.
12. Successful DRG cultures have been established from fetal sheep of less than 100 d of gestation and human fetal material following therapeutic termination at 10–13 wk gestation.
13. Compared to mouse tissue, fetal human and sheep DRG are substantially bigger, more deeply embedded between the vertebrae and the nerve roots, and are more heavily myelinated at the harvest times listed in **Note 12**.
14. Approximately 30–40 DRG are routinely collected from each fetal sheep. Due to the very limited availability of human fetal tissue, cultures have been successfully prepared from as few as 12–20 DRG.
15. Handle all human tissue using universal precautions and treat with bleach before discarding. Wash all instruments in detergent and autoclave after use.
16. Use of the antimitotic agent cytosine arabinoside, enables better visualisation of axonal outgrowth by limiting the growth of fibroblasts and Schwann cells.
17. Poly-L-lysine (50 μg/mL) can also be used as a substrate for DRG culture. In explant culture DRG appear less adherent on poly-L-lysine-coated plates and maintain a more spherical form.
18. When lentivirus stocks are added to DRG cultures at an estimated MOI of 1–2, transduction efficiency is low, and transgene expression poorly maintained. Transduction efficiency of sensory neurons is similar at MOIs of 20, 100 and 500; however, at the higher MOIs, EGFP fluorescence intensity is increased. Initial transduction does not become evident until approx 3 d after the addition of virus to the culture medium. EGFP expression in neurons continues to increase over time, with expression throughout the axons visible by d 7. Maximum expression is observed at approximately d 10.
19. EGFP-expressing cells can be distinguished by the following cellular morphologies: Schwann cells (bipolar and spindle shaped), neurons (spherical cell bodies, fine axonal projections), and fibroblast-like cells (large, flattened, and irregular shape).
20. EGFP expression can be maintained for 28 d; however, expression decreases as the cultures begin to degenerate (21 d onward).

21. Newborn mouse DRG explants can be microinjected to increase delivery of vector to cells within the explant interstitium. The use of low MOIs results in poor transduction rates, and high titer stocks are therefore preferred given the limited volumes that can be microinjected (1–2 μL). Explants can be microinjected after they have been established in culture for 4–5 d. To make microinjection needles, prepare glass capillaries using a vertical puller and microforge to a diameter of 2–3 μm. Load vector into glass capillaries using loading pipets. Attach glass capillary to a microinjector (e.g., Narashigi, Tokyo, Japan). Position capillary directly above the DRG and lower carefully to puncture the DRG. Inject vector slowly using compressed air (400 to 1,000 hPa). Leave the needle in position for 2 min before gently withdrawing.
22. Transduction events are visible 3 d after injection. Expression is maintained for 28 d, the limit of the culture system.
23. Using wash times of at least 5 min and increasing the volume of the wash buffer can reduce background problems with antibody staining.
24. With DAPI counterstaining, three distinct nuclear morphologies are discernible Schwann cells (small fusiform nuclei), neurons (small spherical nuclei), and fibroblast-like cells (large flattened nuclei).
25. Intensity of EGFP fluorescence is reduced after antibody staining.

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References

1. Mazarakis, N. D., Azzouz, M., Rohll, J. B., et al. (2001) Rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery. *Hum. Mol. Genet.* **10,** 2109–2121.
2. Desmaris, N., Bosch, A., Salaun, C., et al. (2001) Production and neurotropism of lentivirus vectors pseudotyped with lyssavirus envelope glycoproteins. *Mol. Ther.* **4,** 149–156.
3. Naldini, L. (1998) Lentiviruses as gene transfer agents for delivery to non-dividing cells. *Curr. Opin. Biotechnol.* **9,** 457–463.
4. Naldini, L., Blömer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. USA* **93,** 11,382–11,388.
5. Blömer, U., Naldini, L., Kafri, T., Trono, D., Verma, I. M., and Gage, F. H. (1997) Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J. Virol.* **71,** 6641–6649.
6. Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L., and Trono, D. (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat. Biotechnol.* **15,** 871–875.

7. Scott, S. A. (ed.) (1992) Sensory neurons: diversity, development, and plasticity. Oxford University Press, New York.
8. Fleming, J., Ginn, S. L., Weinberger, R. P., et al. (2001) Adeno-associated virus and lentivirus vectors mediate efficient and sustained transduction of cultured mouse and human dorsal root ganglia sensory neurons. *Hum. Gene Ther.* **12,** 77–86.
9. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36,** 59–74.
10. Foley, G. E., Lazarus, H., Farber, S., Geren Uzman, B., Boone, B. A., and McCarthy, R. E. (1965) Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer* **18,** 522–529.
11. Naldini, L., Blömer, U., Gallay, P., et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.
12. Johnson, M. I. and Bunge, R. P. (1997) Primary cell cultures of peripheral and central neurons and glia, in *Protocols for Neural Cell Culture* (Federoff, S. and Richardson, A., eds.), Humana Press, Totowa, NJ, pp. 51–75.
13. Lycke, E., Kristensson, K., Svennerholm, B., Vahlne, A., and Ziegler, R. (1984) Uptake and transport of herpes simplex virus in neurites of rat dorsal root ganglia cells in culture. *J. Gen. Virol.* **65,** 55–64.
14. Wilcox, C. L., Smith, R. L., Freed, C. R., and Johnson, E. M., Jr. (1990) Nerve growth factor-dependence of herpes simplex virus latency in peripheral sympathetic and sensory neurons in vitro. *J. Neurosci.* **10,** 1268–1275.
15. Penfold, M. E., Armati, P., and Cunningham, A. L. (1994) Axonal transport of herpes simplex virions to epidermal cells: evidence for a specialized mode of virus transport and assembly. *Proc. Natl. Acad. Sci. USA* **91,** 6529–6533.
16. Kafri, T., Blömer, U., Peterson, D. A., Gage, F. H., and Verma, I. M. (1997) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat. Genet.* **17,** 314–317.
17. Barry, S. C., Harder, B., Brzezinski, M., Flint, L. Y., Seppen, J., and Osborne, W. R. (2001) Lentivirus vectors encoding both central polypurine tract and post-transcriptional regulatory element provide enhanced transduction and transgene expression. *Hum. Gene Ther.* **12,** 1103–1108.
18. Dull, T., Zufferey, R., Kelly, M., et al. (1998) A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* **72,** 8463–8471.
19. Zufferey, R., Dull, T., Mandel, R. J., et al. (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* **72,** 9873–9880.

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Cardiomyocytes

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1. Introduction

Conventional ways of introducing genes into cells, such as calcium phosphate transfections and liposome or lipofectamine-mediated methods, work poorly in cardiomyocytes. They can be useful for gene reporter studies, but are of little value when a gene needs to be introduced into a large number of cardiac cells. Therefore, viral vectors are used to achieve this goal. Type 5 adenoviruses work well in cardiomyocytes, but they are hard to produce, and moreover, their immunogenicity prevents their use in long-term in vivo experiments ***(1)***. The adeno-associated virus is suitable for in vivo myocardial gene transduction because of its low or absent immunogenic potential ***(1)***, but its use for in vitro studies is limited by the low gene expression achieved (less then 10% in cardiomyocytes) **(2)**. The classical retroviral systems are of no practical value either for studies of growth-arrested cardiomyocytes owing to the requirement of M phase for integration with host genomic DNA. Lentiviruses, however, can enter the nucleus even without mitosis ***(3)***, and recently, a new variant of third generation lentivirus ("advanced" generation) has been described ***(4)*** in which sequences of the pol gene of human immunodeficiency virus type 1 (HIV-1), central polypurine tract (cPPT) and of the posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE) have been inserted. The cPPT is a *cis* element that has been associated with increased gene expression in growth-arrested human hematopoietic progenitor cells ***(4)***. This type of virus is suitable for efficient and relatively easy introduction of genes into cardiomyocytes for both in vitro and in vivo studies.

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2. Materials

2.1. Primary Cardiomyocyte Culture

1. Rat pups: the culture must be done utilizing pups from day 1 to 3 postpartum.
2. 10X Digestion Buffer: 116 m*M* NaCl, 5m*M* KCl, 0.9 m*M* NaH_2PO_4, 0.7 m*M* $MgSO_4$, 5 m*M* glucose, 20 m*M* N-2-hydroxyethylpipevozine-N-2-etholesulfonic acid (HEPES), pH 7.3 to 7.4. Make fresh and filter (*see* **Note 1**).
3. Collagenase Type 2 (Worthington Biochemical Corp., Lakewood, NJ): keep at 4°C.
4. Pancreatin (Sigma, St. Louis, MO): store at –20°C. This is dissolved with the collagenase in 1X digestion buffer to make the enzyme solution used to dissociate the cells of the heart.
5. Percoll (Sigma): used after the digestion steps to purify cardiomyocytes from noncardiomyocytes that make up approx 70% of the cells of the heart. The stock solution must be made fresh, diluting 9 vols Percoll with 1 vol 10X digestion buffer. The top Percoll is made by diluting 9 vols stock solution with 11 vols 1X digestion buffer, while the bottom Percoll is made by diluting 13 vols stock solution with 7 vols 1X digestion buffer. Just before use, place 3 mL of bottom Percoll into a 15-mL conical centrifuge tube and carefully layer 4 mL of top Percoll on top of this (*see* **Note 2**). Make one tube for every 10 to 15 hearts used.
6. Gelatin (bovine skin type B) (Sigma) is used to treat cultureware as cardiomyocytes do not readily attach to untreated surfaces. Warm culture-grade water and dissolve gelatin to make a 1% solution, which is then autoclaved and can be kept at room temperature (RT) for several weeks. Cover the surface of the culture-ware with the gelatin solution, aspirate off, and allow to dry for at least 1 h before seeding cells. This must be done on the day of culture.
7. Dulbecco's modified Eagle medium (DMEM) (GIBCO-Invitrogen Corp., Carlsbad, CA): choose a low glucose (1000 mg/mL) formulation.
8. Medium 199 (GIBCO): choose a formulation containing sodium bicarbonate. Mix with DMEM in the following ratio: 4 vols DMEM to 1 vol Medium 199. Store at 4°C.
9. HS (Horse Serum) (GIBCO): decomplement the serum by heating to 56°C for 30 min. Store in aliquots at –20°C.
10. FBS (fetal bovine serum) (Euroclone, Wetherby, West Yorkshire, UK): choose an endotoxin free serum. Decomplement and store aliquots at –20°C.
11. L-glutamine.
12. Penicillin/streptomycin.
13. Cytosine-β-D-arabinofuranoside (Sigma): added to the medium to inhibit the growth of any noncardiomyocytes that are not removed by the Percoll gradient. Dissolve in water to make a 1 m*M* (100X) stock solution. Store aliquots at –20°C.
14. Jacketed Spinner Flask (Wheaton, Millville, NJ).

2.2. Materials Needed for Infection and Analyses

1. Polybrene: dissolve the powder in cell culture-grade water to a final concentration of 8 mg/mL. This makes a 2000X stock solution. Store in aliquots at –20°C.

2. Trypsin-ethylenediaminetetraacetic acid (EDTA): ensure that it contains a pH indicator.
3. PBS (phosphate-buffered saline) (GIBCO): used ice cold to wash cells.
4. 2% serum/PBS; 2% paraformaldeheide (PFA)/PBS; 1% Triton X-100/PBS.
5. Cytofluorimeter and CellQuest (Becton Dickinson, Franklin Lakes, NJ) or WindMDI (Microsoft, Redmond, WA) software.

2.3. Other Cell Cultures

1. 293T cells: (also called 293tsA1609ne) are used to produce the virus. They are derived from 293 cells (a continuous human embryonic kidney cell line), transformed by sheared Type 5 adenovirus DNA and by transfection with the tsA 1609 mutant gene of SV40 Large T Antigen and the *neo*^{r} gene of *E. coli* and are grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, L-glutamine (50 U/mL), and penicillin/streptomycin (50 U/mL). 293T cells are semiadherent and have a high growth rate, but they are very sensitive to culture density. In fact, they lose their transfectability and virus production capacity if cultured too concentrated in the plate. Therefore, the cells should be subcultured every two days using trypsin-EDTA. These cells are very transfectable and produce viruses very efficiently. They work like a complex translation system in which all viral proteins are synthesized in large amounts; the assembled virus is then released into the medium by budding. The backbone of the vector constructs contain an SV40 origin of replication; therefore these cells, which express Large T Protein, are good recipients for episomal replication of the plasmid used.
2. IMDM (GIBCO): prepare from powder and use within two months.
3. TF1 cells: these are a human erythroleukemia cell line, used for testing the transduction efficiency of lentiviral vector preparations since they are easily infected. They are grown in RPMI medium, supplemented with 10% FBS and 10 ng/mL granulocyte/macrophage colony-stimulating factor (GM-CSF).
4. GM-CSF (Preprotech, London, UK): resuspend the powder in IMDM/0.1% bovine serum albumin (BSA). Store aliquots at –20°C. Thawed aliquots can be kept at 4°C for 1 month.

3. Methods

3.1. Recovery of Neonatal Rat Cardiomyocytes

Neonatal rat cardiomyocytes are obtained utilizing a modification of an original protocol *(5–7)* as follows.

3.1.1. Preparation of Enzyme Solution

1. Weigh out collagenase (80 U/mL enzyme solution) and pancreatin (0.6 mg/mL enzyme solution) (*see* **Note 3**).
2. Add enzymes to 1X digestion buffer (keep on ice). The volume of enzyme solution depends on the number of hearts used (2 to 3 litters should be adequate)

and the number of digestions; use 0.3 mL enzyme solution/heart/digestion. A total of seven to eight digestions should be sufficient to digest most of the heart tissue.

3.1.2. Collect Hearts

1. Place 1- to 3-d-old rat pups in a container.
2. Decapitate pup while holding it behind its front legs.
3. Place pup on its back under the hood and make a midline incision through the sternum.
4. Press down on the body of the pup with large curved forceps to pop the heart up through the incision.
5. Remove the heart and place in a 100-mm Petri dish containing 1X digestion buffer.
6. Repeat **steps 2–5** for all of the pups.

3.1.3. Prepare Hearts for Digestion

1. Trim the atria away from the rest of the heart (*see* **Note 4**).
2. Place the remainder of the heart in a clean dish containing digestion buffer and mince into small pieces. Once all of the hearts have been minced, pour the pieces into the spinner flask (*see* **Note 5**).
3. Pump appropriately heated water through the outer jacket of the spinner flask so that the temperature of the inner chamber is kept at 37°C.

3.1.4. Digest Minced Ventricles with Multiple Rounds of Digestions

1. Aspirate and discard the digestion buffer that was poured into the spinner flask along with the minced tissue.
2. Add the appropriate amount of enzyme solution (0.3 mL/heart).
3. Incubate for 6 min (*see* **Note 6**).
4. Collect and discard this first digestion because it contains mainly broken cells and blood. Leave the undigested pieces of heart behind in the spinner flask.
5. Add the appropriate amount of fresh enzyme solution to the undigested tissue and incubate for 20 min (*see* **Note 7**).
6. Collect the digest from the spinner flask, leaving the undigested pieces of heart behind, and pipet into a 15-mL centrifuge tube containing 3 mL of HS to inactivate the enzymes (*see* **Note 8**).
7. Repeat **steps 5** and **6** until almost all of the tissue has been digested (*see* **Note 9**).

3.1.5. Collect Digests and Pool the Cells

1. At the end of each digestion, centrifuge the collected digest at 400*g* in a swinging-bucket benchtop centrifuge for 6 min to pellet cells (*see* **Note 10**).
2. Discard the supernatant, resuspend the pellet with a small quantity of HS, and pool in a 50-mL tube. Keep the pool in the incubator with the cap loose.

3.1.6. Enrich for Cardiomyocytes on a Percoll Density Gradient (see ***Note 11***)

1. At the end of the multiple rounds of digestions, the pooled cells are centrifuged at 400*g* for 10 min.
2. Discard the supernatant and resuspend the pellet in 40 mL 1X digestion buffer.
3. Centrifuge again at 400*g* for 10 min (*see* **Note 12**).
4. The washed pellet is then resuspended in 1X digestion buffer (2–3 mL for every 10 to 15 hearts).
5. Layer 2-3 mL of the resuspended cells gently onto the Percoll gradient, formed as described in **Subheading 2.1.5.**, making sure that the two layers are not disrupted.
6. The tubes are centrifuged at 800*g* for 30 min (*see* **Note 13**).
7. Enriched cardiomyocytes, found at the Percoll top/bottom interface (*see* **Note 14**), are removed from each Percoll gradient and pooled in a 50-mL tube containing 1X digestion buffer.
8. Centrifuge at 400*g* for 10 min.
9. Discard the supernatant and resuspend the pellet in medium.
10. Repeat centrifugation and resuspend the pellet in a small quantity of medium.

3.1.7. Seed Cells and Culture

1. Count (using trypan blue) and seed cells (using the viable-cell count) (*see* **Note 15**) on gelatin-treated 24-well plates containing plating medium, made as follows: DMEM-Medium199 (4:1) supplemented with 10% HS, 5% fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin/streptomycin. Add cytosine-β-D-arabinofuranoside to inhibit the growth of any remaining contaminating cells.
2. Change the medium 24 h later. Wash cells with PBS and add maintenance medium. This is identical to plating medium but contains a reduced amount of HS (5%), making the total serum content now 10%. Allow the cells to grow for a further day before transduction.

3.2. Analyze the Recovered Cell Population by Fluorescence-Activated Cell Sorter (FACS)

1. Aspirate medium, wash cells with PBS, and then remove PBS.
2. Add 200 μL of trypsin-EDTA and incubate at 37°C (*see* **Note 16**).
3. Add 200 μL of ice-cold medium containing serum, and pipet into a 15-mL centrifuge tube containing 1 mL ice-cold medium containing serum.
4. Centrifuge at 600*g* for 5 min at 4°C.
5. Tip out the supernatant.
6. Resuspend the pellet with cold 2% serum/PBS and wash twice.
7. Resuspend in 1 mL of 2% PFA/PBS and incubate at 4°C for 10 min to fix cells.
8. Add 50 μL of 1% Triton X-100/PBS (0.05% final concentration) and incubate at 4°C for a further 10 min to permeabilize cells.
9. Add 1 mL of 2% serum/PBS and centrifuge.

10. Wash cells in 2% serum/PBS.
11. Incubate cells with a cardiomyocyte-specific primary antibody, such as an antitroponin I antibody, for 30 min at 4°C. Dilute antibody in 2% serum/PBS.
12. Wash cells and incubate with a tetramethylrhodamine isothiocyanate-labeled secondary antibody for 30 min at 4°C in the dark.
13. Wash cells and resuspend in PBS.
14. Analyze at FACS using the appropriate controls to set gates (*see* **Note 17**).

3.3. Transduction of Cardiomyocytes In Vitro

Transduction of human cardiomyocytes has been successfully carried out by using either of the lentiviral transfer vectors depicted in **Fig. 1**. Lentiviral particles are generated by transient transfection in 293T cells and titrated on TF 1 cells, as already described in previous chapters of this book. The plasmids used to produce viral particles are: (1) the packaging construct, pCMVΔR8.74, designed to provide the HIV-1 proteins needed to produce the virus particle; (2) the envelope-coding plasmid, pMD.G, for pseudotyping the virion with VSV-G (vesicular stomatitis virus glycoprotein-G), and (3) the self-inactivating (SIN) transfer vector plasmid pRRL.cPPT.hPGK.EGFP.WPRE or pRRL.cPPT.CMV.EGFP.WPRE **(Fig. 1)**. The transfer vector contains the enhanced green fluorescent protein (EGFP) that can be used to sort infected cells. It has an additional DNA sequence of 118 bp (cPPT) situated before the GFP cassette, taken from the *pol* gene of HIV-1, which seems to be required in *cis* for the passage of the viral genome across the nuclear membrane. Another critical sequence is the WPRE at the 3′ end of the expression cassette, which confers stability to the mRNA.

1. Plate the cardiomyocytes 2 d before transduction at 2.5×10^4 cells/well in 24-well plates. These cells do not replicate and can be cultured only for a short time.
2. Thaw the viral supernatant in a bath at 37°C and add polybrene to a final concentration of 4 μg/mL.
3. Remove the medium from the cells to be infected and replace with the viral supernatant.
4. Centrifuge the plates for 45 min in a Beckman (Beckman Instruments Inc., Palo Also, CA) GS-6KR centrifuge, at 500*g* at 32°C.
5. Afterward, keep the cells in a 5% CO_2 incubator for either a further 1 h and 15 min at 32°C or overnight at 37°C.
6. Wash cells twice with cold PBS and add fresh medium. Cells can be analyzed 48 h later for the expression of the gene marker (*see* **Note 18**). Cardiomyocytes result as susceptible to lentiviral transduction as TF1 cells **(Fig. 2)**. Viruses containing either the cytomegalovirus (CMV) or the human phosphoglycerate kinase (hPGK) promoter work well in cardiomyocytes, even if hPGK seems slightly more efficient **(Fig. 3)**.

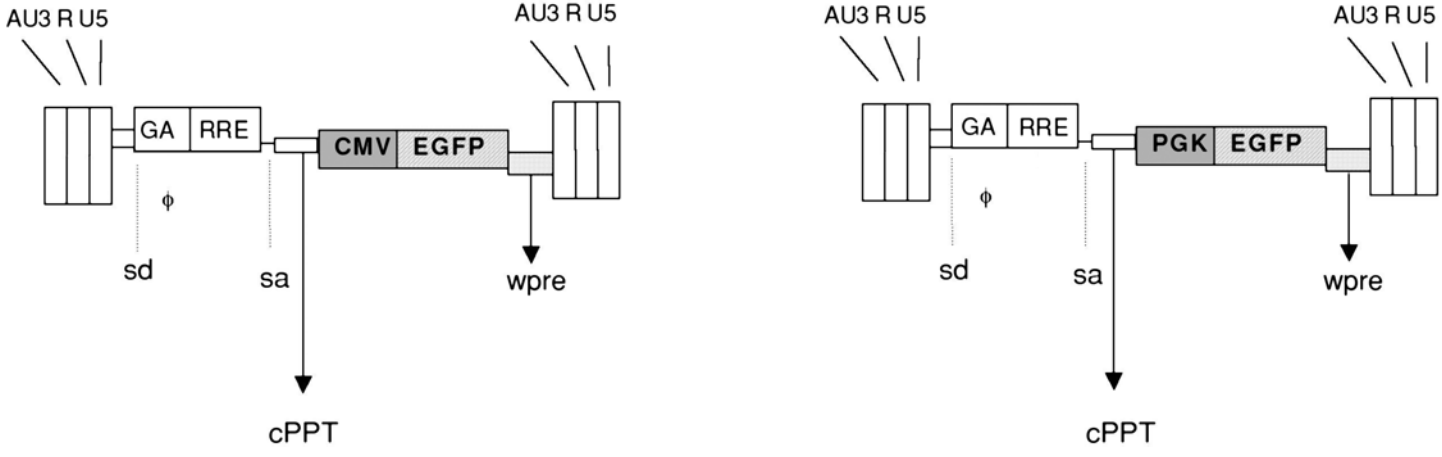

Fig. 1. Diagram showing the transfer vector with either the CMV or hPGK promoter.

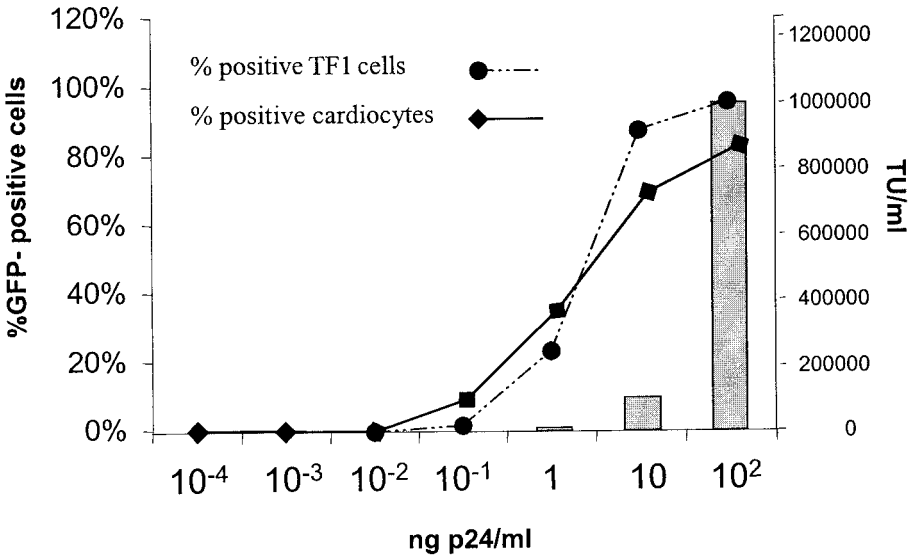

Fig. 2. Graph showing the transduction of cardiomyocytes and TF 1 cells (25,000 cells/mL) with increasing dilutions of lentiviral supernatant (10^2 to 10^4 ng p24/mL) as analyzed by FACS. The histogram indicates the number of transduction units/ml for each dilution. A good viral supernatant should contain $>10^4$ TU/ng p24.

3.4. Infection of Myocardial Cells In Vivo

Lentiviruses can be used also in vivo. A 250-fold more concentrated supernatant than the regular viral stock needs to be used. The injection method performed is a modification of the method established by Hajjar et al. ***(8)*** and further modified by Ikeda et al. ***(9)***.

1. Anesthetize the animal with a mixture of ketamine hydrochloride (Sigma, 50 mg/Kg) and xylazine (Sigma, 10 mg/Kg).
2. Orally intubate and ventilate.
3. Cool the animals with ice bags and monitor the temperature with a thermistor catheter.
4. When the animal's temperature is above 20°C, make an anterior thoracotomy.
5. Exteriorize the heart and place a 7.0 suture on the apex of the left ventricle.
6. Gently introduce a 22 G catheter containing 200 μL of virus solution into the left ventricle. The aortic root and pulmonary artery are identified and the catheter advanced through the left ventricle to the aortic root.
7. Gently clamp the aorta and pulmonary artery distal to the site of the catheter and inject the lentiviral solution. The clamp is maintained for 15 s allowing the solution to circulate down the coronary arteries.

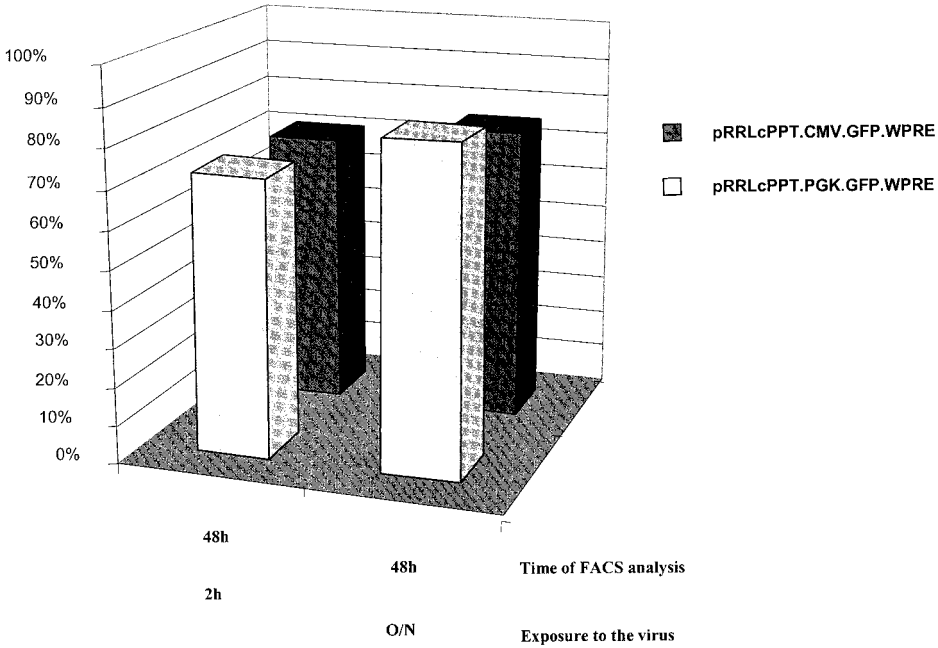

Fig. 3. Comparison of in vitro transduction of cardiomyocytes with the CMV and hPGK promoters.

8. Remove the clamp and catheter, reduce the pneumothorax and close the chest.
9. Transfer the animal back to its cage to recover. The same procedure is followed for sham-operated rats, but the catheter is filled with saline.

4. Notes

1. It is a good idea to check the pH again after diluting to the 1X solution.
2. The addition of a few grains of phenol red powder to the bottom Percoll will ease the visualization of the two gradients. If the top Percoll is overlaid properly, the Percoll top/bottom interface will be seen as a dark line. Do not handle the tubes roughly as this will cause mixing of the two layers.
3. Enzyme concentrations may need to be adjusted to obtain the best yield and viability.
4. This can be done arbitrarily by simply removing the top quarter of the heart. Atria contain a high percentage of noncardiomyocytes, so it is a good idea to remove them.
5. Alternatively, the digestions can be carried out in a 50-mL centrifuge tube in a rocking heated water-bath.
6. The spinner flask should be placed on a magnetic stirrer and the velocity kept to a minimum since cardiomyocytes are very sensitive to shear forces.

7. Incubation times may need to be adjusted to obtain the best yield and viability.
8. The enzyme solution will become cloudy as cells are released into it. An increased yield may be obtained by trituration of the minced tissue with a 5-ml pipet just before collecting the digest.
9. The tissue may not be completely digested after all the rounds of digestion. Each laboratory should adjust the number of digestions with respect to the amount of cells required.
10. Treat culture-ware now, between the digestions. This treatment will allow enough time for the gelatin to dry before seeding.
11. Preplating of the digest may be preferred to separation with Percoll because it is much easier to perform and does not necessitate any additional reagents. The pooled digested cells are washed in plating medium and seeded onto untreated 100-mm Petri dishes for 1 h (this step may be repeated a second time; again, optimal time and numbers of preplating steps should be established by each laboratory). Fibroblasts and other contaminants will adhere to the untreated surface, leaving the cardiomyocytes freefloating in the medium. The medium is collected, centrifuged and the cells plated onto gelatin-treated culture-ware. Both methods have their advantages and disadvantages, and it is up to personal taste which is used.
12. Prepare the Percoll gradients during this second wash step.
13. Move the tubes gently to avoid mixing the different layers. Start the centrifugation (brake off) by accelerating slowly to 300*g*, then quickly to 800*g*. A smoothly operating centrifuge is imperative in obtaining sharply separated bands.
14. After centrifugation, two bands of cells should be observable. The top band, seen at the Percoll top/digest interface, contains mainly noncardiomyocytes (fibroblasts, endothelia, and so on), and this should be removed first by careful aspiration. The lower ring, seen at the Percoll top/bottom interface, contains enriched cardiomyocytes. A pellet of erythrocytes will be seen at the bottom of the tube.
15. Viable cell counts range from 0.5 to 2 × 10^6 cells. Anything above 1 × 10^6 is considered good.
16. Cardiomyocytes are harder to trypsinize than many other cell types. Incubate with trypsin-EDTA at 37°C for 1 min. Longer incubation times may be necessary. Strike the plate or pipet vigorously to aid release of cardiomyocytes.
17. Cultures should contain more than 95% cardiomyocytes.
18. One major problem with virus particles containing VSV-G protein in the envelope arises when cells are overinfected. In fact, apart from decreasing viability, pseudotyping transduction artifacts become evident. This is due to the introduction of already translated protein that is transported within the viral membrane envelope directly into the cell. Thus, it is necessary to perform dose-response experiments carefully. Moreover, an integrase-negative virus can be used to quantify this phenomenon with dose-response curves, analyzing the cells at FACS at various times. Fluorescence due to pseudotyping artifacts are detectable for only 5–7 d.

References

1. Hajjar, R. J., del Monte, F., Matsui, T., and Rosenzweig, A. (2000) Prospects for gene therapy for heart failure. *Circ. Res.* **86,** 616–621.
2. Svensson, E. C., Marshall, D. J., Woodard, K., et al. (1999) Efficient and stable transduction of cardiomyocytes after intramyocardial injection or intracoronary perfusion with recombinant adeno-associated virus vectors. *Circulation* **99,** 201–205.
3. Kay, M. A., Glorioso, J. C., and Naldini, L. (2001) Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat. Med.* **7,** 33–40.
4. Follenzi, A., Ailles, L. E., Bakovic, S., Geuna, M., and Naldini, L. (2000) Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat. Genet.* **25,** 217–222.
5. Sen, A., Dunnmon, P., Henderson, S. A., Gerard, R. D., and Chien, K. R. (1988) Terminally differentiated neonatal rat myocardial cells proliferate and maintain specific differentiated functions following expression of SV40 large T antigen. *J. Biol. Chem.* **263,** 19,132–19,136.
6. De Luca, A., Sargiacomo, M., Puca, A., et al. (2000) Characterization of caveolae from rat heart: localization of postreceptor signal transduction molecules and their rearrangement after norepinephrine stimulation. *J. Cell Biochem.* **77,** 529–539.
7. Condorelli, G., Borello, U., De Angelis, L., et al. (2001) Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardium regeneration. *Proc. Natl. Acad. Sci. USA* **98,** 10,733–10,738.
8. Hajjar, R. J., Schmidt, U., Matsui, T., et al. (1998) Modulation of ventricular function through gene transfer in vivo. *Proc. Natl. Acad. Sci. USA* **95,** 5251–5256.
9. Ikeda, Y., Gu, Y., Iwanaga, Y., et al. (2002) Restoration of deficient membrane proteins in the cardiomyopathic hamster by in vivo cardiac gene transfer. *Circulation* **105,** 502–508.

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Airway Epithelia

Larry G. Johnson, Scott H. Randell, and John C. Olsen

1. Introduction

Cystic fibrosis (CF) is a common inherited disorder affecting a variety of epithelial tissues. The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) that lead to abnormal secretions, recurrent infection and inflammation, bronchiectasis, and premature death. Because airways disease is the major cause of morbidity and mortality in cystic fibrosis, gene therapy efforts have focused on luminal delivery of vector to the airways of CF patients. Retroviruses are attractive as a gene transfer vector system since integration of the wild-type CFTR cDNA into the host genome may lead to long-term expression and perhaps, a cure. However, simple retroviruses are limited as vectors for airway gene transfer by the low rates of epithelial cell proliferation in human airways (~0.1–0.2%) combined with the traditionally low titers. Advances in vector design and production have improved titers, and the development of human and animal lentiviruses may help overcome the requirement for cell proliferation. These developments have raised hopes for retroviral approaches for treatment of CF lung disease.

The best-known lentiviruses are the human immunodeficiency viruses types 1 and 2 (HIV-1 and 2) and simian immunodeficiency viruses (SIV). HIV, feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV) have each been developed as gene transfer vectors for cystic fibrosis. Lentiviral vectors have been shown to transduce a variety of nondividing cell types in vitro and in vivo ***(1–9)***. HIV, EIAV, and FIV vectors have been shown to efficiently transduce aphidicolin-treated (growth-arrested) airway epithelial cells in vitro ***(6–8,10)***.

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The envelope proteins of lentiviral vectors bind to cell surface receptors to facilitate entry with subsequent reverse transcription, nuclear import, and integration into the host chromosome culminating in gene expression. To evaluate the polarity of lentiviral transduction, vescicular stomatitis virus glycoprotein-G (VSV-G) pseudotyped vectors have been applied to either the apical or the basolateral surfaces of polarized well-differentiated (WD) human airway epithelial (HAE) cells. Thirty-fold greater transduction efficiency was observed in vitro when HIV VSV-G vectors were applied to the basolateral surface as compared to apical application of vector ***(11)***. Goldman and colleagues demonstrated that luminally applied HIV (VSV-G) vectors failed to transduce WD primary HAE cells in bronchial xenografts ***(11)***. Similar data have evolved for EIAV pseudotyped with VSV-G ***(11a)***, and for amphotropic- and VSV-G-enveloped FIV vectors ***(7,10)***. These data suggest that, while lentiviral vectors can transduce nondividing cells, the receptors for uptake and entry of amphotropic and VSV-G pseudotyped MLV and lentiviral vectors are predominantly localized to the basolateral membrane of polarized epithelial cells.

Two strategies have been proposed to overcome the lack of apical membrane receptor expression: (1) host modification with injury models and agents that permeabilize tight junctions to increase vector access to basolateral membrane receptors and basal cells ***(11)*** and (2) targeting the apical membrane of polarized airway epithelia by pseudotyping lentiviruses with envelope proteins from other viruses that bind and enter across the apical membrane.

Injury models and transient permeabilization of intercellular junctions are methods by which host cells have been modified to increase access to the basolateral receptors. Johnson et al. exposed mice to sulfur dioxide (SO_2) inhalation to increase vector delivery to the basolateral surface and/or basal cells of murine tracheas. Induction of airway cell proliferation peaked at 24 h postinhalation with no significant increase in proliferation occurring in the first 12 h after inhalation ***(12)***. SO_2 also caused a dose-related denuding of the surface epithelium leaving the basal cell layer intact. In regions less severely injured by SO_2, permeability was increased through the tight junctions ***(12)***. Using this model, mice were exposed to SO_2 for 3 h at 500 ppm, and a HIV (VSV-G) vector was delivered onto the nasal or tracheal epithelia of rodents ***(8)***. Efficient reporter gene transfer was observed in nasal and tracheal airway epithelia, whereas no gene transfer was detected in the nasal or tracheal airway epithelia of sham (air)-exposed controls ***(8,12)***. Gene transfer to murine trachea was also more efficient following SO_2 exposure when vector was administered on the same day of exposure (~7% of cells transduced) and when cell proliferation was not increased, as compared to the day after SO_2 inhalation (2% transduction of cells) at which time peak airway cell proliferation occurs.

This finding is consistent with preferential transduction of nondividing cells ***(8)***. These data demonstrate that HIV vectors can efficiently transduce airway epithelia in vivo when access to receptors on the basolateral membrane is increased by injury.

Transient permeabilization of tight junctions is another method by which to increase vector delivery the basolateral membrane and basal cells. Nonprimate lentiviral vectors have been coadministered with calcium chelators to airways to increase vector access to basolateral receptors, presumably by modulating paracellular permeability ***(7,10)***. Wang et al. applied FIV-CFTR vectors in combination with hypotonic solution and ethylene glycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid (EGTA) to the apical membrane of polarized WD CF airway epithelial cells ***(7,10)***. Restoration of chloride secretory function was achieved following infection with FIV-CFTR in primary CF airway epithelial cells that was similar to levels of correction achieved with an adenoviral (Ad)CFTR vector. The correction of chloride secretory function mediated by FIV-CFTR persisted for up to six months in culture, whereas the AdCFTR-mediated Cl- correction had resolved by 21 d after transduction. Subsequently, these investigators demonstrated relatively efficient transduction of ciliated and nonciliated cells within rabbit airways in vivo comprising approx 5% of airway cells infected with vector at d 5 and decreasing to approx 2.5% of cells at 6 wk ***(7)***. The mechanism of attenuation of transgene expression was not evaluated. Nevertheless, FIV-CFTR vectors offer promise as vectors for gene therapy of cystic fibrosis.

Olsen and colleagues have developed a mammalian lentiviral vector based on EIAV ***(6)***. As compared to HIV-1, EIAV has a much simpler genome. Horses infected with the wild-type EIAV virus typically survive the infection (~95%), and wild-type EIAV does not replicate in human cells. Olsen demonstrated efficient transduction of WD primary HAE cells after apical application of EIAV (VSV-G) β-galactosidase (lacZ) vector in the presence of ethylenediaminetetraacetic acid (EDTA) or EGTA to increase access to basolateral receptors by permeabilizing the tight junction. Persistence of transgene expression in vitro lasted for up to 24 d posttransduction, the longest period tested. In preliminary studies, EIAV vectors with internal promoters have been shown to express reporter genes in murine tracheal airway epithelia following SO_2 injury to increase delivery to basal cells and the basolateral membrane ***(13)***. The efficiency of transduction was dose-related with minimal transduction at vector titers of 10^8 infectious units (i.u.)/mL, whereas vectors with approx 10^{10} i.u./mL yielded transduction efficiencies that were significantly greater than those observed at lower EIAV lentiviral titers ***(13)***.

Targeting lentiviral vectors to receptors on the apical membrane is an alternative strategy to increase airway gene transfer. The usual paradigm for

retroviral targeting is the generation of pseudotypes from envelope proteins of other viruses that target specific cell types. Toward this goal, investigators have screened enveloped viruses for their ability to infect WD HAE cells from the apical surface. Although some efforts have focused on common respiratory viruses, others have also included nonrespiratory viruses. Of the respiratory viruses considered as candidates for pseudotyping, respiratory syncytial virus (RSV), human corona virus, and influenza virus have received the most attention. Wild-type and replication-competent recombinant RSV have been shown to infect or enter cells across the apical membrane of WD HAE cells ***(14,15)***. Human corona virus 229E (HcoV229E) has also been shown to bind and enter across the apical membrane of WD HAE cells ***(16)***. However, no successful lentiviral pseudotypes derived from the Env proteins of these respiratory viruses have been reported.

Influenza A virus subtype H2N2/Japan/305/57 has been shown to preferentially infect the apical, rather than the basolateral, membrane of polarized WD HAE cells, whereas apical infection of HAE cells with subtypes H1N1 and H3N2 was inefficient ***(17)***. The binding of the H2N2 subtype appeared to be specific for sialic acid α2,3-gal residues, suggesting that these sialic acid residues may serve as an apical membrane receptor for targeting of lentiviral pseudotypes. Recently, Morse et al. have shown in preliminary studies that coexpression of influenza M2 and neuraminic acid (NA) proteins in producer cells enhances titers of influenza hemagglutinin pseudotyped EIAV vectors ***(18)***. Moreover, these HA/M2/NA chimeric pseudotyped vectors transduced polarized WD HAE cells following luminal application.

The Env proteins of other viruses have also been considered. In a preliminary study, FIV-based vectors were pseudotyped with amphotropic, xenotropic, VSV-G, RD-114, 10A1, ecotropic, gibbon ape leukemia virus (GALV), Marburg, and Ebola envelope glycoproteins by transient transfection techniques. Only FIV vectors pseudotyped with the Marburg virus envelope glycoprotein efficiently transduced WD HAE cells following luminal application ***(19)***.

Kobinger and colleagues further explored the potential of this family of viruses, known as the Filoviridae, to target the apical membrane of airway epithelia ***(20)***. Filoviridae are enveloped, nonsegmented negative sense RNA viruses, which include Ebola virus and Marburg virus among its members. To identify viral envelopes capable of mediating apical transduction of polarized air-liquid interface cultures, HIV vectors were pseudotyped with murine leukemia virus (MLV), influenza-hemagglutinin, RSV F and G proteins, Mokola, Ebola Reston (Ebo-R), or Ebola Zaire (EboZ) envelopes. Concentrated stocks of EboZ, but not EboR or other pseudotypes, efficiently transduced polarized HAE cells following apical application with gene transfer in up to 70% of cells as compared to up to 40% of cells following basolateral

transduction. EboZ-pseudotyped HIV vectors also transduced WD HAE cells in xenografts and in freshly excised human tracheal explants. Thus, lentiviral vectors may overcome the limitations of low rates of cellular proliferation, and pseudotyping lentiviral vectors with specific envelope proteins may overcome barriers to titer and the lack of apical membrane receptors on polarized WD HAE cells.

Previously, we have published methods for retroviral infection of primary HAE cells on plastic and for functional characterization of CFTR function in vitro ***(21)***. In this chapter, we focus on methods for lentiviral transduction of polarized, well-differentiated primary human airway epithelia in vitro. An EIAV vector pseudotyped with VSV-G will serve as the prototypical vector for which the methods have been written. However, the methods should also be applicable to HIV, VSV-G, and other pseudotyped lentiviral vectors. Enhancement of luminal gene transfer will be described for host modification approaches shown to be useful for at least one of the currently employed VSV-G pseudotyped lentiviral vectors studied in airways.

2. Materials

2.1. EIAV Lentiviral Vector Production

2.1.1. Culture of 293T Cells and BiG-45 EIAV Packaging Cells

1. Dulbecco's modified Eagle medium, high glucose (DMEM-H), with 4500 mg/L glucose and L-glutamine (Invitrogen, Carlsbad, CA).
2. 293T cells, cultivated in DMEM-H supplemented with 10% fetal bovine serum (FBS) (Invitrogen).
3. BiG-45 cultivated in DMEM-H supplemented with 10% Tet System Approved FBS (Clontech, Palo Alto, CA) (*see* **Note 1**).
4. Penicillin (50 U/mL) and streptomycin (50 μg/mL) as antibiotics.

2.1.2. Production of Lentiviral Vectors

We generally use $CaPO_4$-mediated transfection to generate virus by transient transfection. We make our own reagents, although we have on occasion used the Calcium Phosphate Transfection System from Life Technologies (Carlsbad, CA) with good results.

2.1.2.1. Calcium Phosphate Transfection Reagents

1. 0.5 *M* HEPES (pH 7.1): Dissolve 12 g HEPES (4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid [Roche Applied Science, Indianapolis, IN] in H_2O to a final volume of 100 mL. Adjust pH to 7.1 ± 0.05 with 3 *N* NaOH. Sterilize by filtration using a 0.2-μm filter (*see* **Note 2**).
2. 2 *M* NaCl: Dissolve 11.7 g NaCl in H_2O to a final volume of 0.1 L. Sterilize by filtration using a 0.2-μm filter.

3. 2 *M* $CaCl_2$: Dissolve 29.4 g $CaCl_2$•$2H_2O$ in H_2O to a final volume of 0.1 L. Sterilize by filtration using a 0.2-μm filter.
4. 150 m*M* phosphate buffer (pH 7.0): Dissolve 4.02 g Na_2HPO_4•$7H_2O$ in H_2O to a final volume of 100 mL. Dissolve 2.08 g NaH_2PO_4•H_2O in H2O to a final volume of 100 mL. Adjust 42.3 mL NaH_2PO_4 solution to pH 7.0 with 57.7 mL of the Na_2HPO_4 solution. Sterilize by filtration using a 0.2-μm filter.
5. 2X HEPES-buffered saline (HBS) solution: Mix 1.53 mL sterile H_2O, 200 μL 0.5 *M* HEPES (pH 7.1), 250 μL 2 *M* NaCl, and 20 μL 150 m*M* phosphate buffer (pH 7.0). Make up fresh 2X HBS solution on the day of transfection.

2.1.2.2. Induction Reagents

1. 500 m*M* sodium butyrate (50X stock): Dissolve 0.55 g (Sigma, St. Louis, MO) in H_2O to a final volume of 10 mL. Sterilize by filtration using a 0.2-μm filter. Store at –20°C.
2. 1.5 mg/mL doxycycline (1000X stock in 95% ethanol, obtained from Sigma). Store in dark (light sensitive) at –20°C.

2.1.2.3. Concentration of Virus by Low Speed Centrifugation

1. Formulation buffer: 19.75 m*M* Tris-HCl (pH 7.0), 40 mg/mL lactose, 37.5 m*M* NaCl, 5 μg/mL protamine sulfate. Sterilize by filtration using a 0.2-μm filter. Store at 4°C.
2. Centrifuge tubes and adapters for the Beckman SW 28 rotor: 30-mL Konical tubes (Beckman Coulter, Fullerton, CA) and adapters.

2.2. Primary Culture of Human Airway Epithelial Cells

1. Phosphate-buffered saline (PBS): Dissolve 8 g NaCl, 0.2 g KCl, 2.16 g Na_2HPO_4•$7H_2O$, and 0.2 g KH_2PO_4 in 800 mL distilled deionized H_2O. Adjust to pH 7.2 and bring to 1 liter final volume.
2. 1.0 mg/mL protease Type XIV (Sigma) plus 0.1 mg/mL deoxyribonuclease I (Sigma) in Jolik's minimum essential medium (JMEM) (0.2-μm filter sterilized).
3. Trypan blue (0.4%) in PBS (Sigma).
4. Purified collagen (Vitrogen-100, Cohesion, Palo Alto, CA).
5. Collagen-coated plastic tissue culture dishes (*see* **Note 3**).
6. Trace Elements
   Make stock solutions of all trace elements in tissue culture grade H_2O as specified below.

Selenium ($NaSeO_3$)	52.0 mg in 100 mL	3.0 m*M*
Manganese ($MnCl_2$•$4H_2O$)	1.26 mg in 100 mL	0.1 m*M*
Silicone (Na_2SiO_3•$9H_2O$)	1.42 g in 100 mL	50 m*M*
Molybdenum (NH_4)$6Mo_7O_{24}$•$4H_2O$)	12.4 mg in 100 mL	100 μ*M*
Vanadium (NH_4VO_3)	5.9 mg in 100 mL	0.5 m*M*
Nickel ($NiSO_4$•$6H_2O$)	1.3 mg in 100 mL	0.1 m*M*
Tin ($SnCl_2$•$2H_2O$)	1.1 mg in 100 mL	50 μ*M*

Add 1 mL of each stock and 1 ml of 36% HCl to 992 mL distilled deionized H_2O and filter-sterilize.

7. Divalent Cation stock
   Make stock solution as specified below:

Ferrous Sulfate ($FeSO_4$•$7H_2O$)	0.042 g (1.5×10^{-4} *M*)
Magnesium Chloride ($MgCl_2$•$6H_2O$)	12.20 g (6.0×10^{-2} *M*)
Calcium Chloride ($CaCl_2$•$2H_2O$)	0.411 g (2.8×10^{-3} *M*)
Hydrochloric Acid (concentrated)	0.5 mL

   Bring volume to 1 liter with tissue culture grade H_2O, and filter-sterilize.
8. Bovine pituitary extract (BPE) (*see* **Note 4**).
9. Transwell-col tissue culture substrates (0.4-μ*M* pore size, Corning Costar, Acton, MA).
10. JMEM calcium-free and magnesium-free (Life Technologies).
11. DMEM-H.
12. Growth medium for primary airway epithelial cells on plastic (*see* **Note 5**): LHC Basal media (Biofluids, Rockville, MD) supplemented with hormones and growth factors (**Table 1**). Penicillin (50 U/mL), streptomycin (50 μg/mL), and gentamicin (50 μg/mL), amphotericin B (250 μg/mL) are added as antibiotics.
13. Air–liquid interface (ALI) medium. Prepare a 1 : 1 mixture of LHC basal medium and DMEM-H. Add growth factors and hormones used for the growth medium, but reduce epidermal growth factor (EGF) concentration reduced to 0.5 ng/ml and omit amphotericin B and gentamicin. Filter-sterilize.

2.3. Reagents for Gene Transfer to Undifferentiated and Polarized Well-Differentiated HAE Cells

1. Polybrene (Sigma) stock of 4 mg/mL and sterilized by passing through a 0.2-μm syringe filter.
2. EGTA, 0.1 *M* stock. Dissolve 9.51 g in 200 mL PBS. Adjust pH to 7.4. Bring to 250 mL final volume and sterilize through a 0.2-μm filter.

3. Methods

3.1. Production of Lentiviral Vectors Based on EIAV

EIAV vectors can be efficiently produced by three-plasmid transfection of 293T cells (similar to other vector production systems discussed in this volume) or from recently developed EIAV packaging cell lines. We use the BiG-45 packaging cell line, which is a 293-based cell line that has been modified to express the gag-pol and rev proteins from EIAV and the VSV-G envelope glycoprotein. These cells are useful for producing EIAV lentiviral vectors by a single plasmid transfection of the gene transfer vector (method follows) or alternately can be stably modified with the gene transfer vector to create a full-fledged vector producing cell line. These cells were derived from a subclone (293.101) of the human embryonic kidney cell line, 293, in two steps. First, stable transfection of 293.101 cells with the helper plasmid pEV53B resulted in

Table 1
Hormone and Growth Factors for Growth Medium

Additive	Stock Prep	Stock Conc.	Final Conc.	Storage
Insulin (INS)	250 mg/47.5 mL H_2O + 2.5 mL acetic acid	5 mg/mL	5 μg/mL	–20°C
Hydrocortisone (HC)	1) 10 mg/13.8 mL 70% ETOH	0.72 mg/mL		–20°C
	2) Dilute 1:10 in PBS	0.072 mg/mL	0.072 μg/mL	
Epidermal Growth Factor (EGF)	100 μg/4 mL PBS	25 μg/mL	25 ng/mL ALI conc =0.5 ng/mL	–20°C
Triiodothyronine (T_3)	1) 6.5 mg/5 mL 0.2 *M* NaOH	1×10^{-5} *M*	1×10^{-8} *M*	–20°C
	2) Add 5 mL H_2O			–20°C
	3) Dilute 1:100 in H_2O			–20°C
Transferrin (T_f)	500 mg/50 mL PBS	10 mg/mL	10 μg/mL	–20°C
Epinephrine (EPI)	1) 30 mg/5 mL 0.1 *N* HCl	0.6 mg/mL	0.6 μg/mL	–20°C
	2) QS to 50 mL with H_2O			
Phosphoethanolamine (Phosphoeth)	7 mg/100 mL PBS	0.5 m*M*	0.5 μ*M*	–20°C
Ethanolamine (Eth)	3.1 μL/100 mL PBS	0.5 m*M*, 0.031 mg/mL	0.5 μ*M*, 0.031 μg/mL	23°C
Bovine Pituitary Extract (BPE)	BPE stock solution (*see* **Subheading 2.2.**)	100X	1X	–20°C
Bovine Serum Albumin (BSA)	150 mg/mL in F12(1X)	150 mg/mL in F12(1X)	0.5 mg/mL	–20°C
Calcium Chloride ($CaCl_2$)	8.88 g/L in ddH_2O	0.08 *M*	0.08 m*M*	23°C
Trace Elements	Trace Elements stock solution (*see* **Subheading 2.2.**)	100X	1X	4°C
Divalent Cation Stock	*see* **Subheading 2.2.**	100X	1X	23°C
Zinc Sulfate Heptahydrate ($ZnSO_4 \cdot 7H_2O$)	0.863 g/L in ddH_2O Filter-sterilize.	3×10^{-3} *M*	3×10^{-6} *M*	–20°C
Retinoic Acid (RA)	1) 12.5 mg/40 mL ethanol for 1×10^{-3} *M* stock.	5×10^{-5} *M*	5×10^{-8} *M*	–20°C
	2) Add 3 mL 1×10^{-3} stock to 50 mL 12 mg/mL endotoxin-free BSA in PBS.			
	3) Bring to final vol. 60 mL w/PBS.			

B-241 cells. B-241 cells were then modified to express VSV-G under regulated expression by the tetracycline repressor. Cells are routinely maintained on poly-L-lysine-coated dishes. Cells are passaged 1:4 every fourth day. Do not allow the cells to overgrow. New cultures of BiG-45 cells are thawed every 1–2 mo.

3.1.1. Preparation of VSV-G Pseudotyped EIAV Vectors Following Transient Transfection of 293T Cells or BiG-45 Cells

The following recipe is for 10-cm plates. The procedure can be modified by changing volumes/quantities proportionally for scale-up or scale-down. Note that the expression of EIAV viral proteins in BiG-45 cells is inducible by both doxycycline and sodium butyrate. Sodium butyrate treatment also results in increased vector yield from 293T cells. Omission of sodium butyrate will result in reduced vector titers.

1. Seed cells to obtain approx 80% confluence the next day. For 293T cells, the plating density is about 5×10^6 cells/100-mm polystyrene tissue culture dish. BiG-45 cells are plated at approx $1.0–1.2 \times 10^7$ cells/100-mm tissue culture dish. DNA transfer will be most efficient if the cells are just subconfluent at the time of transfection. Incubate overnight at 37°C in a humidified incubator with 5% CO_2 (*see* **Note 6**).
2. On the day of transfection, make up the 2X HBS solution.
3. For a three-plasmid transfection of 293T cells: In polystyrene tubes, mix 12 μg each of the gag-pol expression vector (pEV53B) and the gene transfer vector and 6 μg of the envelope expression vector (pCI-VSV-G) with H_2O to give a final volume of 262.5 μL. For a single plasmid transfection of BiG-45 cells, mix 12 μg of the gene transfer vector DNA with H_2O to give a final volume of 262.5 μL. To the DNA solution, add 37.5 μL 2 *M* $CaCl_2$ (final concentration of 250 m*M* $CaCl_2$).
4. For each transfection, aliquot 300 μL 2X HBS solution into a polystyrene tube. Add the 300 μL DNA/$CaCl_2$ mixture dropwise, then bubble air through the solution 5–10 times with a 1 mL pipet tip. Alternately, bubble air through a 1 mL pipet during addition.
5. Incubate the mixture 20–30 min at room temperature.
6. Remove medium from cells and replace with 6 mL fresh medium. Then add the 600 μL sample to the cells, dropwise. Swirl the plate gently to mix. Incubate overnight at 37°C in a humidified incubator with 5% CO_2.
7. Next day, remove the medium. Add 6 mL of fresh growth medium per plate containing 10 m*M* sodium butyrate per plate. For induction of VSV-G expression in BiG-45 cells, include 1.5 μg/mL doxycycline. Incubate 24 h at 37°C in a humidified incubator with 5% CO_2.
8. Next day: Swirl the plates gently, then remove cell supernatant containing virus. Filter virus through a syringe filter containing 0.22-μm cellulose acetate or

polyethersulfone (PES) membranes. Store virus in aliquots at –70°C. For large batches (100 mL to 750 mL), remove large cell debris by centrifugation (100*g*, 10 min) prior to filtration through a Corning 0.22-μm PES membrane filter system (Fisher Scientific, Pittsburgh, PA, 250 mL or 500 mL).

9. If desired, a second harvest of vector can be collected. In this case, add 6 mL of fresh growth medium without sodium butyrate to each culture dish (include 1.5 μg/mL doxycycline if using BiG-45 cells). Incubate 24 h at 37°C in a humidified incubator with 5% CO_2 and harvest virus as described in **step 8**.

3.1.2. Concentration of EIAV Vectors by Low Speed Centrifugation

The advantage of concentrating lentiviruses by low speed centrifugation is that it provides a more gentle alternative than ultracentrifugation which can result in the loss of certain labile envelope glycoproteins ***(22)***. We have used this approach for successfully concentrating EIAV vectors pseudotyped with the VSV-G, murine amphotropic, Jaagsiekte sheep retrovirus, and influenza hemagglutinin (HA) envelopes. The following protocol has been used with the Beckman SW 28 rotor using the conically shaped Beckman brand Konical tubes (30 mL capacity) to pellet virus, but should be adaptable for use with other rotors and tubes. Using this protocol, it is possible to process up to 180 mL of virus-containing medium. For larger volumes (250 mL to 3 L) the Sorvall (Newton, CT) H6000A rotor has been used successfully ***(22)***.

1. Prerinse polyallomer tubes with approx 20 mL aliquots of acetone (three times), 95% ethanol (twice), and tissue culture grade H_2O (three times). Allow tubes to drain in tissue culture hood.
2. Fill tubes with filtered virus from the producer cells. Place tubes into rotor buckets. (If the Beckman Konical tubes are used, be sure to use special adapters to prevent tube collapse.)
3. Pellet the virus using a swinging bucket rotor (SW 28) at 5000*g* for 20 h at 4°C.
4. Decant supernatant and remove excess liquid from sides of tube above pellet with UV-sterilized Kimwipes or other lintless laboratory wipers.
5. Suspend virus in a small volume of formulation buffer and use for immediate gene transfer or store in aliquots at –70°C.

3.2. Primary Culture

3.2.1. Tissue Procurement

1. Obtain excess excised nasal or bronchial tissues from fresh surgical specimens (e.g., nasal polyps or lobes of the lung; *see* **Note 7**).
2. Remove and discard loose connective tissue by blunt and sharp dissection.
3. Incubate in excess volume (1:10 tissue:medium) of chilled (4°C) JMEM containing penicillin (50 U/mL), streptomycin (50 mg/mL), and gentamicin (50 μg/mL) for 2–24 h.

3.2.2. Isolation of HAE Cells

1. Rinse tissue with fresh JMEM plus antibiotics and incubate in 1:10 v/v (tissue:medium) 0.1% protease Type XIV plus 1.0 μg/mL deoxyribonuclease I in JMEM at 4°C for 16–48 h on a rocker shaker (*see* **Note 8**).
2. Transfer the tissue to a sterile 10-cm culture dish and gently scrape cells from the mucosal side of the tissue with the convex edge of a scalpel blade. Then rinse the cells away from the tissue with the protease solution.
3. Add FBS to cells suspensions from **steps 1** and **2** to 10% (v/v) to neutralize the protease.
4. Remove tissue pieces, pellet cell suspension (500*g* for 5 min at 4°C).
5. Wash cells with JMEM containing 10% FBS and pellet at 500*g* for 5 min.
6. Stain aliquot of cells with an equal volume of trypan blue solution and count viable and dead cells in a hemacytometer.
7. Resuspend cells in growth medium to desired volume.

3.2.3. Primary Culture of Undifferentiated Cells on Plastic

1. Seed isolated cells onto collagen-coated 10-cm plastic dishes at a density of 1×10^6 cells/dish in growth medium.
2. Feed cells 24 h after seeding with fresh medium and every other day thereafter.
3. On culture days 5 and 6, wash cells with PBS, incubate cells in 0.1% trypsin/1.0 m*M* EDTA at 37°C until they dissociate (~5 to 10 min), then add 20% by volume soybean trypsin inhibitor (1 mg/mL in serum-free DMEM). Harvest cells and pellet at 500*g* for 5 min.
4. Wash cells with 5 mL fresh medium, repellet, and seed cells at 2.5×10^5 cells/well of a 6-well plate in growth medium to create passage 1 (p1) primary cells.
5. Feed cells after 24 h and every 2–3 d until used for studies.

3.2.4. Primary Culture of Polarized WD Airway Epithelial Cells

1. Seed p0 or p1 cells at a density of 2×10^5 cells/12 mm (0.4-μm pore size) Transwell-Col insert in ALI medium (*see* **Note 9**).
2. When cultures reach confluence (~5–7 d), aspirate medium gently from the apical surface creating an air-liquid interface.
3. Maintain in culture by feeding every 1–2 d for an additional 2–3 wk.
4. Confirm polarization by measurement of transepithelial resistance (R_t) beginning on days 18 through 21 in culture with an ohmmeter (EVOM, World Precision Instruments, Sarasota, FL) (*see* **Note 10**).
5. Confirm the presence of WD phenotype by the presence of cilia on greater than 10–20% of the cells upon visualization by phase contrast microscopy (typically 3–4 wk in culture).

3.3. Gene Transfer to Undifferentiated Primary HAE Cells Grown on Plastic

Primary HAE cells are a good host for lentiviral vectors containing either the amphotropic envelope or pseudotyped with the VSV-G envelope glycoprotein

with gene transfer efficiencies as high as 50–100% of primary cells transduced after a single infection.

1. Plate cells in plastic tissue culture dishes as described in **Subheading 3.2.3.**
2. At 24 h after plating, infect cells by replacing medium with 0.5–1 mL lentiviral vector. Add polybrene to 8 μg/mL and return cells to incubator.
3. After 2 h, remove virus and replace with growth medium. Analyze cells for expression 48–72 h after infection.

3.4. Gene Transfer to Polarized WD Airway Cells

Polarized WD primary HAE cells are resistant to luminal transduction by VSV-G pseudotyped, amphotropic enveloped, and GALV pseudotyped vectors. Methods that increase access of vectors to the basolateral surface where receptors appear to be expressed at higher density, appear to increase gene transfer efficiency following luminal application of vector. Several techniques have been developed to enhance gene transfer. These include basolateral infection, injury with pipet tip, and modulating paracellular permeability.

3.4.1. Basolateral Infection

1. Seed and grow primary human cells on transwell-col (12 mm) inserts until they develop cilia and R_t as described in **Subheading 3.2.**
2. Remove transwells from tissue plate and invert in 10-cm dish.
3. Gently aspirate medium from basolateral surface and apply 100 μL vector containing 8 μg polybrene to the basolateral surface of inverted transwell.
4. Incubate for 2 h at 37°C, then return transwells to 12-well plate in the upright position.
5. Harvest cells and test for gene expression 72 h later.

3.4.2. In Vitro Pipet Injury Model

This model permits the investigator to assess the ability to infect from the apical or basolateral surface in the same culture. A pipet is used to scratch the epithelial surface such that transduction by vectors that preferentially transduce basolateral surfaces occurs along the line of injury that exposes the basolateral surface, but not in the uninjured areas of the epithelium.

1. Score the luminal surface of a polarized well differentiated HAE cells with a sterile Pasteur pipet across the diameter of the insert.
2. Immediately apply 100–200 μL vector to the lumen of the culture in the presence of 8 μg/mL polybrene and incubate at 37°C for 2 h.
3. Aspirate medium from surface and evaluate for gene expression by histochemistry, immunolabeling, or fluorescence microscopy 72 h later. Gene expression will occur along the line of injury if the vector efficiently transduces basolaterally localized receptors.

3.4.3. Modulating Paracellular Permeability

Calcium chelating agents and medium chain fatty acids have been shown to enhance gene transfer to polarized airway epithelia mediated by Ad, Adenovirus-associated vectors (AAV), and lentiviral vectors. Of these the chelating agents EGTA and EDTA have been most effective with lentiviral vectors. EGTA has been routinely used with both pretreatment and coadministration of vector. We describe strategies for EGTA- or EDTA-mediated enhancement of lentiviral vector gene transfer.

3.4.3.1. Pretreatment Strategies

1. Measure baseline R_t, then add EGTA (10 m*M*) in Ca^{2+}-free HEPES-buffered Ringer solution to the luminal surface of polarized WD cells with Ca^{2+}-containing HEPES-buffered Ringer solution on the basolateral surface (*see* **Note 11**).
2. Measure R_t every 5–10 min until R_t falls by 90% or more of baseline resistance. Residual resistance should be approx 50–80 Ohms-cm^2 after correction for the blank. Generally takes more than 45 min (*see* **Note 12**).
3. Wash cells with medium bilaterally and feed basolateral surface with 1 mL ALI medium.
4. Apply vector containing 8 μg/mL polybrene in medium to lumen of culture and incubate 2 h at 37°C in tissue culture incubator.
5. Aspirate medium, wash cells twice, and maintain in culture for 72 h or until desired for gene expression analysis.

3.4.3.2. Coadministration with Vector

1. Dilute concentrated EGTA or EDTA stock (0.1–0.5 m*M*) admixed with vector to final concentration 10 m*M*.
2. Wash the lumen of WD HAE cells with PBS or medium, then apply vector containing 8 μg/mL polybrene to apical surface.
3. Incubate at 37°C for 2 h, then remove vector and wash cells twice with medium, then feed cells with fresh medium.
4. Assay for gene expression at 72 h or when desired.

4. Notes

1. BiG-45 cells are maintained on tissue culture plates coated with poly-L-lysine (PLL) to improve adherence of cells to the plate. PLL coating solution is made up by adding 16 mL of tissue culture grade poly-L-lysine (Sigma, St. Louis, MO) to 500 mL tissue culture grade H_2O. To coat 10-cm tissue culture plates, 6 mL of PLL coating solution is added to the plate and incubated for 2 h at room temperature. The PLL solution is removed, and the remaining PLL is allowed to collect near one edge by raising one side of the plate. After removing the solution with a Pasteur pipet, the plates are allowed to dry completely in a tissue culture hood for several hours.

2. It is prudent to make up several HEPES stocks from pH 7.05 to pH 7.2, and test them functionally.
3. Collagen-coated plastic tissue culture dishes are prepared as follows. Dilute sterile concentrated purified collagen 1:75 in sterile H_2O. Apply 0.5 mL/well to 12-well culture plate, 1.0 mL/well to 6-well culture plate, or 4 mL/well to 10-cm plate. Incubate from 2 h to overnight at 37°C. Remove collagen by aspiration and air dry in tissue culture hood. UV sterilize under tissue culture hood for 10–15 min. Seal plates with parafilm and store at 4°C for up to 6 wk.
4. We prepare our own BPE stocks. However, BPE can also be purchased commercially (Clonetics, San Diego, CA). Thaw mature unprocessed bovine pituitaries (Pel Freeze, Rogers, AZ) at 4°C. Drain blood and rinse pituitaries with chilled PBS. Add 200 g of tissue to 400 mL cold PBS and mince in Hamilton-Beach (Washington, NC) Blendmaster-7-speed blender for 10 min. Transfer blended solution into 500 mL Beckman centrifuge canisters (Beckman JA-10 Rotor) and centrifuge at 5000*g* for 10 min at 4°C. Combine supernatants and aliquot. Before use, respin aliquot at 5000*g* for 10 min at 4°C and pass supernatant through a 0.4-μm filter.
5. We prepare our own growth medium ***(23,24)***, but a similar commercial product is available (BEGM, Clonetics, San Diego, CA). For culture of airway epithelial cells from the lungs or cystic fibrosis subjects, additional antibiotics are required ***(24)***.
6. For 293T cells, as with BiG-45 cells, higher vector yields are obtained if poly-L-lysine-coated plates are used for transfection. We attribute this to better adherence of the cells to the plate during medium changing.
7. The use of human tissues generally requires approval from your local institutional review board (IRB) for the protection of human subjects. Although some uses may be exempt from review, we recommend that all investigators submit a protocol for review. An exemption, if appropriate, will be granted by the IRB.
8. Epithelial cell yield is greater after 48 h of protease digestion than after 24 h, without significant loss of viability. By 72 h of protease digestion, cell viability has decreased significantly.
9. Other commercially available permeable substrates can be used for culture of polarized HAE cells including Millipore filters (Bedford, MA) and Transwell-Clear (Corning Costar).
10. The EVOM electrodes should be sterilized before use by soaking in 70% ethanol and equilibrated in ALI medium prior to use. All measurements should be made in a tissue culture hood at room temperature. Measure the resistance across a blank T-Col and record. Subtract the blank resistance from measured resistance across HAE cells on a T-cols and multiply by the surface area to generate the actual R_t. When the culture has fully differentiated into an epithelial sheet with tight junctions, polarized HAE cells will have a R_t of 200 to 1200 Ohms-cm^2. Typically p0 and p1 cells have a higher resistance than p2 cells.
11. Some investigators apply a hypotonic Ca^{2+}-free solution (10 m*M* HEPES, pH 7.4) containing EGTA or EDTA to the luminal bath.

12. Bilateral application of EGTA or EDTA leads to a rapid fall in resistance (within minutes). However, epithelium may lift off from the substrate if chelators are left on the basolateral surface too long.

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References3

1. Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. USA* **93,** 11,382–11,388.
2. Naldini, L., Blomer, U., Gallay, P., et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.
3. Naldini, L. (1998) Lentiviruses as gene transfer agents for delivery to non-dividing cells. *Curr. Opin. Biotechnol.* **9,** 457–463.
4. Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L., and Trono, D. (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat. Biotechnol.* **15,** 871–875.
5. Zufferey, R., Dull, T., Mandel, R. J., et al. (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* **72,** 9873–9880.
6. Olsen, J. C. (1998) Gene transfer vectors derived from equine infectious anemia virus. *Gene Ther.* **5,** 1481–1487.
7. Wang, G., Slepushkin, V., Zabner, J., et al. (1999) Feline immunodeficiency virus vectors persistently transduce nondividing airway epithelia and correct the cystic fibrosis defect. *J. Clin. Invest.* **104,** R55–R62.
8. Johnson, L. G., Olsen, J. C., Naldini, L., and Boucher, R. C. (2000) Pseudotyped human lentiviral vector-mediated gene transfer to airway epithelia in vivo. *Gene Ther.* **7,** 568–574.
9. Wang, G., Sinn, P. L., and McCray, P. B., Jr. (2000) Development of retroviral vectors for gene transfer to airway epithelia. *Curr. Opin. Mol. Ther.* **2,** 497–506.
10. Wang, G., Zabner, J., Deering, C., et al. (2000) Increasing epithelial junction permeability enhances gene transfer to airway epithelia In vivo. *Am. J. Respir. Cell Mol. Biol.* **22,** 129–138.
11. Goldman, M. J., Lee, P. S., Yang, J. S., and Wilson, J. M. (1997) Lentiviral vectors for gene therapy of cystic fibrosis. *Hum. Gene Ther.* **8,** 2261–2268.

11a. Olsen, J. C., Fu, J., and Patel, M. (1998) Lentiviral vector-mediated gene transfer to polarized epithelia. *Ped. Pulmonol.* **(Suppl. 17),** 268.

12. Johnson, L. G., Mewshaw, J. P., Ni, H., Friedmann, T., Boucher, R. C., and Olsen, J. C. (1998) Effect of host modification and age on airway epithelial gene transfer mediated by a murine leukemia virus-derived vector. *J. Virol.* **72,** 8861–8872.
13. Olsen, J. C., Patel, M., and Rohll, J. B. (2000) An inducible first generation stable packaging cell line for equine lentivrial vectors. *Mol. Ther.* **1,** 315.
14. Zhang, L., Peeples, M. E., Boucher, R. C., Collins, P. L., and Pickles, R. J. (2002) Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. *J. Virol.* **76,** 5654–5666.
15. Morse, K. M. and Olsen, J. C. (2000) Respiratory synctial virus infection of cultured well differentiated airway epithelial cells from cystic fibrosis patients. *Ped. Pulmonol.* **20 (Suppl.),** 229–230.
16. Wang, G., Deering, C., Macke, M., et al. (2000) Human coronavirus 229E infects polarized airway epithelia from the apical surface. *J. Virol.* **74,** 9234–9239.
17. Slepushkin, V. A., Staber, P. D., Wang, G., McCray, P. B., Jr., and Davidson, B. L. (2001) Infection of human airway epithelia with H1N1, H2N2, and H3N2 influenza A virus strains. *Mol. Ther.* **3,** 395–402.
18. Morse, K. M., Patel, M., and Olsen, J. C. (2002) Optimizing influenza hemagglutinin pseudotyping of equine lentiviral vectors. *Mol. Ther.* **5,** S38.
19. Sinn, P. L., Jolly, D., Sauter, S., et al. (2000) Pseudotyping feline immunodeficiency (fiv)-based vectors to target the apical surface of differentiated human airway epithelia. *Ped. Pulmonol.* **20 (Suppl.),** 233.
20. Kobinger, G. P., Weiner, D. J., Yu, Q. C., and Wilson, J. M. (2001) Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. *Nat. Biotechnol.* **19,** 225–230.
21. Olsen, J. C., Johnson, L. G., and Yankaskas, J. R. (1996) Methods for use of retroviral vectors for transfer of the CFTR gene to airway epithelium, in *Methods in Molecular Medicine, Gene Therapy Protocols* (Robbins, P., ed.), Humana Press Inc., Totowa, NJ, pp. 153–168.
22. Bowles, N. E., Eisensmith, R. C., Mohuiddin, R., Pyron, M., and Woo, S. L. (1996) A simple and efficient method for the concentration and purification of recombinant retrovirus for increased hepatocyte transduction in vivo. *Hum. Gene Ther.* **7,** 1735–1742.
23. Bernacki, S. H., Nelson, A. L., Abdullah, L., et al. (1999) Mucin gene expression during differentiation of human airway epithelia in vitro. Muc4 and muc5b are strongly induced. *Am. J. Respir. Cell Mol. Biol.* **20,** 595–604.
24. Randell, S. H., Walstad, L., Schwab, U. E., Grubb, B. R., and Yankaskas, J. R. (2001) Isolation and culture of airway epithelial cells from chronically infected human lung. *In vitro Cell Biol. Anim.* **37,** 480–490.

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Corneal Cells

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1. Introduction

A mammalian cornea can be maintained in vitro for days *(1,2)*, allowing ex vivo transduction of this tissue prior to transplantation *(3,4)*. Ex vivo transduction of the cornea is useful for studying the efficacy of gene products on inhibition of corneal neovascularization *(4)*, amelioration of corneal inflammatory disease, or promotion of corneal wound healing in animal models. Ex vivo transduction can also be used for transfer of genes into corneal epithelial stem cells *(5)*, thereby allowing genetically modified stem cells to be reimplanted into the limbal area *(6)*, providing a means for long-term gene-replacement (e.g., βig-h3 gene) therapy for various inherited corneal diseases *(7)*.

A normal healthy cornea, consisting of the epithelial, stromal, and endothelial cell layers, maintains a state of transparency. Loss of corneal transparency and subsequent loss or impairment of vision can result from aging, injury, inflammatory or neovascular disease, or an inherited disorder. Approximately 50% of corneal allografts are undertaken as a consequence of primary endothelial dysfunction *(8,9)*. An immune response against endothelial cells of the donor cornea can lead to rejection of the allograft *(10)*. Establishment of a vector that can efficiently transfer genes and prevent allograft rejection to the host corneal button should prove useful in corneal transplant surgery.

When choosing a vector for delivery into ocular tissue, the following criteria should be considered: (1) the ability to transfer efficiently a gene into dividing cells (corneal epithelium) as well as mitotically inactive cells (corneal endothelium); (2) the vector should be nontoxic and noninflammatory to host cells; (3) the vector should provide robust and stable expression of the transgene;

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(4) the vector should be nonreplicative; and (5) consideration of the promoter when appropriate cellular expression of a therapeutic gene is required.

Adenoviral vectors are able to introduce novel genes into corneal endothelial cells; however, the transgene expression is short-lived ***(3,11–13)***. A gene gun system is capable of efficient localized in vivo delivery of DNA (gold microbeads coated with plasmid DNA), albeit with short-term expression, to rabbit corneal epithelial cells ***(14)***. Temporary transgene expression was also noted with electropulse-mediated gene delivery, specifically to rat endothelial cells ***(15)***, and with liposome-mediated transfection of human stromal keratocytes (Stout, J. T., unpublished data).

Of the various methods and vectors that allow transfer of foreign genes into corneal cells, the lentiviral system would appear to have the combined advantages of: (1) efficient transduction, (2) robust levels of expression, (3) early onset of transgene expression, and (4) stable, long-lived expression. Lentiviral-mediated expression of fluorescent green protein within transduced human corneal cells (epithelial, endothelial, and stromal) has been maintained for greater than 60 d with no signs of adverse cellular reaction ***(16)***. The lentivirus system allows for a nontoxic delivery system into dividing (epithelial) and nondividing (endothelial) corneal cells with long-term and stable transgene expression. Thus, the most efficient means of introducing genetic material into corneal tissue as a therapeutic approach to disease is via the lentiviral system.

2. Materials

2.1. Three-Plasmid Cotransfection

1. Lentivirus plasmid DNA (pHR-CMV-Gene of Interest-IRES-EGFP).
2. Vesicular stomatitis virus G glycoprotein (VSV-G) protein expression plasmid (pMD-G).
3. Capsid protein expression plasmid (pCMVΔR8.91).
4. HEPES-buffered saline (HBS): 137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 6 m*M* dextrose, 21 m*M* N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), adjust pH to 7.05 and store at room temperature (RT) for up to 6 mo.
5. 2 *M* $CaCl_2$: autoclave and store at RT.
6. Tissue culture dishes (75-cm^2 flasks).
7. Packaging cell line (293T cells).
8. Culture media: for 293T cells use Dulbecco's modified Eagle's medium (DMEM, high glucose, 2 m*M* L-glutamine) containing 10% fetal calf serum (FCS) and penicillin, streptomycin, amphotericin-B (PSA).
9. Attachment factor (Cascade Biologicals, Portland, OR).
10. 500 m*M* sodium butyrate (filter-sterilize, aliquot, and freeze at 20°C).
11. 1 *M* HEPES (store at 4°C).

2.2. Lentiviral Collection

1. 0.2-μm filter (Nalgene, Rochester, NY).
2. 30-mL syringe.
3. Phosphate-buffered saline (PBS).
4. Oak Ridge centrifuge tubes (Nalgene).
5. 50-mL Falcon tubes.
6. 1.5-mL Eppendorf tubes.

2.3. Lentivirus Quantification

1. p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Zeptometrix, Buffalo, NY).
2. 37°C incubator.
3. 96-well plate reader (HTS 7000 Plus Bio Assay Reader).

2.4. Cell Culture

1. Tissue-culture incubator (37°C 5% CO_2).
2. Tissue culture dishes (6-well plates).
3. Microvascular endothelial cells (MVECs, Cascade Biologicals).
4. Corneal epithelial cells (CECs; Cascade Biologicals).
5. Filter flasks (0.2-μm pore size).
6. Media for MVECs: use Media 131 containing 10% (FCS) and PSA; for CECs use EpiLife containing human corneal growth supplement (HCGS) and PSA (Cascade Biologicals).
7. Eye bank eyes.
8. Optisol (Chiron, Claremont, CA).

2.5. Molecular Verification

1. Trizol (Sigma, St. Louis, MO).
2. Chloroform.
3. Isopropanol.
4. 75% ethanol.
5. Superscript RT Kit (Invitrogen, Carlsbad, CA) contains 10X buffer and dithiothreitol (DTT) (10 m*M*).
6. RedTaq DNA polymerase (Sigma) contains 10X buffer with 1.5 m*M* $MgCl_2$.
7. Deoxynucleotide 5′-triphosphate (dNTPs).
8. Nuclease-free water.

3. Methods

3.1. Three-Plasmid Cotransfection

1. Prepare a 75-cm^2 flask by coating the inside bottom of the flask with 3 mL of attachment factor and place in an incubator for 30 min (*see* **Note 1**).
2. Remove the attachment factor and seed 1×10^5 293T cells to the flask. Add 7 mL of DMEM media and incubate at 37°C with 5% CO_2 (*see* **Note 2**).

3. Grow to 50–80% confluence with media changes every 2 d (*see* **Note 3**).
4. Prepare a 1.5-mL Eppendorf with the following; 15 μg of lentiviral plasmid (plasmid backbone with gene of interest), 15 μg of ΔR8.9 plasmid, 3 μg of VSV-G plasmid (*see* **Note 4**). Bring the volume to 500 μL with HBS buffer and add 32 μL of 2 *M* $CaCl_2$. Mix thoroughly and incubate at room temperature for 45 min (***17***, *see* **Note 5**).
5. Remove media from the 293T cells and pipet the three-plasmid mix onto the monolayer. Incubate 15 min at room temperature, gently rock flask to redistribute the mixture, and incubate an additional 15 min (*see* **Note 6**).
6. Carefully add 7 mL of DMEM culture media to the cells/DNA and incubate at 37°C for 12–24 h.
7. Carefully remove the media-DNA mix and replace with 7 mL DMEM containing 10 m*M* sodium butyrate and 20 m*M* HEPES. Incubate at 37°C for 24 h (***18***, *see* **Note 7**).
8. Remove and discard the sodium butyrate containing media. Add 7 mL fresh DMEM containing 20 m*M* HEPES to the cells incubate for 24 h and collect media. Repeat this incubation and collection for 3 d.

3.2. Lentivirus Collection

1. Pool the collected media (~21 mL) and filter through a 0.2-μm filter into a fresh Oak Ridge centrifuge tubes (*see* **Note 8**).
2. Centrifuge the virus containing media at 40,000*g* (in a Beckman, Palo Alto, CA, LM-60 ultracentrifuge or equivalent) for 2.5 h.
3. Carefully decant the media from the virus pellet and resuspend in 1 mL of PBS and transfer to a fresh 1.5 mL Eppendorf. Store at –80°C (*see* **Note 9**).

3.3. Viral Quantification

Use the recommended manufacturer's protocol for use of the p24 Gag antigen ELISA kit (*see* **Note 10**).

3.4. Cell Culture

1. Seed a 6-well plate with 1×10^5 of MVEC per well. Incubate at 37°C with 5% CO_2 and change media every other day until near confluent.
2. Remove media and add 1, 10, and 100 μL of resuspended viral preparation into different wells to test varying dilutions of virus for infection activity. Incubate without media for 20 min at 37°C. Add 1–2 mL of media and incubate for 48 h, changing media if necessary.
3. After 48 h, view plate under an UV microscope capable of visualizing enhanced green fluorescent protein (EGFP) to confirm expression of the green fluorescent protein from the marker gene (*see* **Note 11**).
4. Once the virus is proven viable in MVEC, it can then be tested on various cell types, such as corneal epithelial or endothelial cell monolayers. Repeat the above cell culture techniques, as well as performing molecular analysis for the

transgene mRNA. A better ex vivo model for the study of corneal transduction via the lentiviral vector is the corneal button assay.

5. Obtain fresh nonfixed eyes from a local eye bank and dissect out the corneal-scleral button. Immediately transfer the button to Optisol media and store at 4°C for a maximum of 3 d (*see* **Note 12**).
6. Add a desired concentration of lentivirus to the Optisol and maintain overnight at 4°C (*see* **Note 13**).
7. The following day remove the button from the Optisol and transfer it to a Petri dish and cover with fresh EpiLife media; place the dish into 37°C, 5% CO_2. Maintain the culture for 36–48 h, after which the sample may be processed by histology or molecular means.
8. Frozen sections can be used to histologically examine where transduction has occurred. Unstained frozen sections can be readily used to visualize fluorescent proteins.
9. If only the corneal endothelial cells need to be examined from the transduced buttons, these cells can be removed by peeling/teasing away the Descemet's membrane (with attached endothelial cells) using fine pointed forceps.

3.5. Molecular Verification

1. Remove media, add 1 mL of Trizol per 50–100 mg of tissue/cells, and pipet up and down. Crushing cells using a pestle and mortar with the aid of liquid nitrogen may be necessary for the corneal button assay. Transfer the solution to a sterile 1.5-mL Eppendorf tube.
2. Follow manufacturer's protocol to isolate total RNA (Trizol Reagent GIBCO, BRL).
3. Perform a standard reverse transcriptase-polymerase chain reaction (RT-PCR) using oligo (dT) primer for the RT and nested primers specific to EGFP for the PCR. Use a housekeeping gene such as β-actin as a positive control.

4. Notes

1. Attachment factor (AF) is not necessary for the seeding of 293T cells. However, cells are more liable to slough off during transfection and subsequent media collection if AF is not utilized.
2. 1×10^5 cells have consistently yielded appropriate cell populations for transfection within a 48 h time period after seeding.
3. A 50–80% confluence yields the highest transfection. Higher or lower cell populations tend to significantly lower the transfection efficiency.
4. Plasmids should be isolated using an endotoxin-free Maxiprep Kit (Qiagen, Valencia, CA). Higher quality plasmid prep is achieved if the vector is propagated in *E. coli* strain DH5α (GIBCO BRL, Carlsbad, CA). Other *E. coli* strains can release large quantities of carbohydrates upon cell lysis and also have high levels of endonuclease activity, resulting in lower quality and lower yields of DNA.
5. Invert tube once every 10–12 min during the incubation stage. A fine hazy precipitate is consistently observed within 45 min using this method.

6. It is essential to redistribute the mixture *carefully,* since overhandling at this stage can easily detach the cell monolayer, decreasing the transfection rate.
7. Sodium butyrate is not crucial for the transfection but has been shown to enhance foreign DNA expression rates and increases the stability of transfected cells ***(18)***.
8. This filtration step separates cell debris from viral particles contained within the media. This step permits for a purer viral pellet after ultracentrifugation.
9. During this step it is often impossible to see a viral pellet, so it is a good idea to mark the outside of the tube and remove the media carefully either by pouring or by pipetting. Keep in mind the position of the pellet. During resuspension, it is important to thoroughly pipet up and down in order to create a homogeneous viral mix.
10. Any standard capsid antigen ELISA kit can be used to quantify the amount of viral particles isolated. This has been a reliable and cost-effective means of detecting purified virus.
11. The time required to observe fluorescence is cell dependent, it takes 48 h in cultured retinal pigment epithelial (RPE), MVEC, and CEC cells, while trabecular meshwork cells can take from 5 to 7 d for maximum expression (Stout, J. T., unpublished data).
12. Other media can be used in this step, however Optisol is the preferred storage media of corneal buttons prior to transplantation and does not interfere with viral infectivity (personal observation). A minimum of 12-h incubation with virus is required to obtain efficient transduction such that, after transplantation of this transduced button into an animal model of penetrating keratoplasty, expression of the transgene can be detected up to 1 mo after surgery ***(19)***.
13. 10^6 to 10^7 viral particles is adequate at transducing a single button. On average approx 10^6 to 10^7 viral particles per 1 mL of Optisol (or enough Optisol to completely submerge the corneal button) was used to transduce corneal buttons. If more than one button needs to be transduced, all the buttons can be placed together in one well, and then submerged in the Optisol/virus mix.

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References

1. Frueh, B. E. and Bohnke, M. (1995) Corneal grafting of donor tissue preserved for longer than 4 weeks in organ-culture medium. *Cornea* **14,** 463–466.
2. Redbrake, C., Sallas S., and Frantz, A. (1998) Changes in human donor corneas preserved for longer than 4 weeks. *Cornea* **17,** 62–65.
3. Larkin, D. F., Oral, H. B., Ring, C. J., Lemoine, N. R., and George, A. J. (1996) Adenovirus-mediated gene delivery to the corneal endothelium. *Transplantation* **61,** 363–370.

4. Yoken, J., Chen, S. L., McFarland, T., et al. (2002) Production of a lentiviral vector containing an endostatin:kringle-5 fusion gene an inhibitor of post-penetrating keratoplasty neovascularization. *IOVS Presentation* S1868.
5. Bradshaw, J. J., Obritsch, W. F., Cho, B. J., Gregerson, D. S., and Holland, E. J. (1999) Ex vivo transduction of corneal epithelial progenitor cells using a retroviral vector. *IOVS* **40,** 230–235.
6. Tsubota, K., Satake,Y., Kaido, M., et al. (1999) Treatment of severe ocular-surface disorders with corneal epithelial stem-cell transplantation. *N. Eng. J. Med.* **340,** 1697–1703.
7. Munier, F. L., Korvatska, E., Djemai, A., et al. (1997) Kerato-epithelin mutations in four 5q31-linked corneal dystrophies. *Nat. Gen.* **15,** 247–251.
8. Price, F. W., Whitson, W. E., Collins, K. S., and Marks R. G. (1993) Five-year corneal graft survival. *Arch. Ophthalmol.* **111,** 799–805.
9. Vail, A., Gore, S. M., Bradley, B. A., Easty, D. L., and Rogers, C. A. (1993) Corneal transplantation in the United Kingdom and Republic of Ireland. *Br. J. Ophthalmol.* **77,** 650–652.
10. Bourne W. M., Hodge D. O., and Nelson L. R. (1994) Corneal endothelium five years after transplantation. *Am. J. Ophthalmol.* **118,** 185–196.
11. Oral, H. B., Larkin, D. F., Fehervari, Z., et al. (1997) Ex vivo adenovirus-mediated gene transfer and immunomodulatory protein production in human cornea. *Gene Ther.* **4,** 639–647.
12. Fehervari, Z., Rayner, S. A., Oral, H. B., George, A. J., and Larkin, D. F. (1997) Gene transfer to ex vivo stored corneas. *Cornea* **16,** 459–464.
13. Budenz, D. L., Bennett, J., Alonso, L., and Maguire, A. (1995) In vivo gene transfer into murine corneal endothelial and trabecular meshwork cells. *IOVS* **36,** 2211–2215.
14. Tanelian, D. L., Barry, M. A., Johnston, S. A., Le, T., and Smith, G. (1997) Controlled gene gun delivery and expression of DNA within the cornea. *Biotechniques* **23,** 484–488.
15. Oshima, Y., Sakamoto, T., Yamanaka, I., Nishi, T., Ishibashi, T., and Inomata, H. (1998) Targeted gene transfer to corneal endothelium in vivo by electric pulse. *Gene Ther.* **5,** 1347–1354.
16. Wang, X., Appukuttan, B., Ott, S., et al. (2000) Efficient and sustained transgene expression in human corneal cells mediated by a lentiviral vector. *Gene Ther.* **7,** 196–200.
17. Yang, X.-J. (2001) Preparation of recombinant retroviruses, in *Vision Research Protocols* (Rakoczy, P. E., ed.), Humana, Totowa, NJ, pp. 171–190.
18. Gorman, C. M., Howard, B. H., and Reeves, R. (1983) Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res.* **11,** 7631–7648.
19. Murthy, R. C., McFarland, T. J., Yoken, J., et al. (2003) Ex vivo transduction of corneas with a novel fusion gene inhibits angiogenesis and graft failure in a corneal transplantation model. *Invest. Opthalmol. Vis. Sci.*, in press.

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Retinal Tissue

Trevor McFarland, Binoy Appukuttan, and J. Timothy Stout

1. Introduction

Mutations within genes that are specifically expressed within the retina and/or the retinal pigment epithelium (RPE) are associated with the majority of inherited retinal degenerations ***(1)***. Retinal degenerative disorders are highly heterogeneous, both genetically and phenotypically ***(2)***. Different mutations within a single gene (such as rhodopsin) can not only result in different modes of inheritance (autosomal dominant as well as recessive) of the same disease (retinitis pigmentosa), but can also result in different retinal disease (congenital stationary night blindness (CSNB) (3). Numerous mutations within the rhodopsin gene and mutations within other genes in the phototransduction pathway have been detected in patients with retinitis pigmentosa (RP), a progressive degenerative disease that leads to death of rod photoreceptors and results in loss of vision. To date, 41 forms of RP, autosomal dominant, recessive, or X-linked, have been mapped, and 21 genes have so far been identified that carry mutations responsible for the disease process ***(4,5)***. Many retinal degenerative diseases are caused by mutations within genes that are involved in the phototransduction pathway or are key structural components of the outer segments of photoreceptors and are thereby expressed exclusively in photoreceptor cells. However, when altered, genes for RPE65, RPE-retinal G protein-coupled receptor (RGR), cellular retinaldehyde-binding protein (CRALBP), and Best macular dystrophy (VMD2), which are expressed in the RPE and not in rod photoreceptors, can also cause similar retinal dysfunction. The RGR, CRALBP, and RPE65 gene products are involved in the regeneration of 11-*cis*-retinal, the chromophore of rhodopsin, from 11-*trans*-retinal, a photoisomerized form of 11-*cis*-retinal. RGR is an opsinlike protein that

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binds all-*trans*-retinal ***(6)*** and functions to regenerate 11-*cis*-retinal under high and continuous light conditions ***(7)***. Mutations within this gene have been associated with recessive RP and choriodal sclerosis ***(8)***. Mutations within the RPE65 gene can cause autosomal recessive RP or Lebers congenital amaurosis (LCA), a congenital childhood form of RP ***(9,10)***. Disruption of the gene encoding CRALBP, which binds and transports 11-*cis*-retinol, an intermediate product in the pathway of the conversion of 11-*trans*-retinal to 11-*cis*-retinal, can cause recessive RP ***(11)*** and *fundus albipunctatus* with associated *retinitis punctata albescens*, another form of retinal atrophy ***(12)***. Thus, it is clear that mutations within genes expressed in the RPE can be associated with a wide variety of ocular phenotypes.

Currently there are no accepted surgical procedures or medications that will prevent the inexorable loss of vision associated with this plethora of inherited retinal degenerative diseases. The discovery and molecular characterization of the causative genes for many of the genetic eye disorders, and the advent of gene transfer vectors give rise to the possibility of gene replacement therapy. A viral vector system capable of efficient gene transfer into terminally differentiated nondividing cells of the retina is a prerequisite for successful gene replacement therapy of these diseases. One might expect that successful treatment would also depend upon appropriate transcription promoters—the level of gene expression, as well as spatial and temporal considerations may prove important ***(13,14)***.

While retroviral vectors, such as the murine leukemia virus (MLV), are capable of integrating into the genome of the target cells, thus achieving long-term transgene expression, these vectors are unable to transduce efficiently nonproliferating cells, such as hepatocytes and neurons ***(15,16)***. In vivo, adenoviral vectors can transfer foreign DNA into dividing and terminally differentiated cell tissues, such as photoreceptor cells. However, expression of the transgene is transient ***(17,18)*** and is often associated with an immune response causing a reduction in transgene expression ***(19,20)***. Investigators have demonstrated that lentiviral derivatives can be used to produce vectors that deliver genes to nondividing neural tissues with long-term expression.

Primary cultures of RPE and choroidal fibroblasts (CFs), and cultured retinoblastoma cell lines Weri-Rb1 and Y79 were transduced at 95, 92, 20, and 40%, respectively, with lentivirus containing the green fluorescent protein (GFP) reporter gene under the control of the cytomegalovirus (CMV) promoter at a multiplicity of infection (MOI) of 1000 (Stout, J. T., unpublished data). Efficient lentiviral-mediated in vivo transfer of the GFP gene, under the control of the CMV promoter, to rat photoreceptors and RPE cells (as well as Müller and bipolar cells to a lesser efficiency) has been demonstrated ***(21)***. Miyoshi and colleagues demonstrated that GFP, under the control of the bovine

rhodopsin promoter, was expressed specifically and at higher levels in rat photoreceptor cells when compared to the CMV-controlled GFP gene, even though the same number of lentiviral particles were injected for each construct ***(21)***. Myoshi and colleagues also showed that a greater number of retinal cells were transduced in young pups, whereas transgene expression was limited to the site of injection in adult rats, suggesting that multiple injections into the subretinal space are required to achieve thorough transduction of the whole retina in an adult mammal. The above experiments are useful in showing that cell-specific expression can be achieved within the mammalian retina using a gene-specific promoter, even if it is from another species, and transduction efficiency can vary depending on the age of development of the animal/organ.

Given this high level of transduction efficiency, can lentiviral vectors be used to rescue the photoreceptor loss observed in RP? The *rd* mouse is a naturally occurring animal model of photoreceptor degeneration as a result of a mutation in the rod photoreceptor 3′,5′-cyclic guanosine monophosphate (cGMP) phosphodiesterase β subunit (PDEβ) gene ***(22)***. Mutations in the human PDEβ gene, whose protein product is responsible for converting cGMP to GMP in the phototransduction cascade in photoreceptors ***(23)***, are found in patients with autosomal recessive RP ***(24,25)***. Subretinal injections of a lentiviral vector containing a murine PDEβ cDNA under the control of the bovine rhodopsin promoter into newborn *rd* mouse eyes was able to rescue photoreceptor cells from the degeneration process ***(26)***. Thus, it seems likely that lentiviral vectors will be suitable for retinal or RPE gene replacement therapy.

It is of interest to note that the promoter choice is important in the design of therapeutic vectors. Temporal, spatial, and quantitative aspects of gene expression are likely to affect therapeutic efficacy ***(13,14)***. Reporter gene expression was observed in both cones and rod photoreceptors in two independent transgenic mouse models carrying either 2240 bp or 292 bp of the bovine rhodopsin promoter, raising the question of photoreceptor-specific expression ***(27)***. The presence of promoter regulatory sequences, such as the photoreceptor consensus element-1 (PCE-1) ***(28)*** and methylation sites ***(29)***, should also be taken into consideration.

Given the number of different genes and their respective promoters and the number of disease-related genes yet to be discovered, it is essential to test the efficacy of each unique promoter and gene product on human cells prior to animal testing and clinical trials. In vitro and ex vivo human retinal culture models will enable direct testing of potentially therapeutic lentivirus for efficiency of transduction into photoreceptors, RPE, and various other cell types germane to eye disorders, as well as the efficiency of foreign promoter-driven transgene expression levels.

2. Materials

2.1. Three-Plasmid Cotransfection

1. Lentivirus plasmid DNA: (pHR-CMV-Gene of Interest-ires-EGFP), (pHR-Promoter of Interest-EGFP) and (pHR-Promoter of Interest-Gene of Interest).
2. Vesicular stomatitis virus G glycoprotein (VSV-G) protein expression plasmid (pMD-G).
3. Capsid protein expression plasmid (pCMVΔR8.91).
4. HEPES-buffered saline (HBS): 137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 6 m*M* dextrose, 21 m*M* N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), adjust pH to 7.0 and store at room temperature (RT) for up to 6 mo.
5. 2 *M* $CaCl_2$: autoclave and store at RT.
6. Tissue culture dishes (75-cm^2 flasks).
7. Packaging cell line (293T cells).
8. Culture media: For 293T cells use Dulbecco's modified Eagles medium (DMEM, high glucose, 2 m*M* L-glutamine) containing 10% fetal calf serum (FCS) and penicillin, streptomycin, amphotericin-B (PSA).
9. Attachment factor (Cascade Biologicals, Portland, OR).
10. 500 m*M* sodium butyrate (filter-sterilize, aliquot, and freeze at –20°C).
11. 1 *M* HEPES (Store at 4°C).

2.2. Lentiviral Collection

1. 0.2-μm filter (Nalgene, Rochester, NY).
2. 30-mL syringe.
3. Phosphate-buffered saline (PBS).
4. Oak Ridge centrifuge tubes (Nalgene).
5. 50-mL Falcon tubes.
6. 1.5-mL Eppendorf tubes.

2.3. Lentivirus Quantification

1. A p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Zeptometrix, Buffalo, NY).
2. 37°C incubator.
3. 96-well plate reader (HTS 7000 Plus Bio Assay Reader, Wellesley, MA).

2.4. Cell Culture

1. Tissue-culture incubator (37°C 5% CO_2).
2. Tissue culture dishes (6-well plates).
3. Filter flasks (0.2-μm pore size).
4. Human retinoblastoma cell lines Y-79 and Weri-Rb1 obtained from the American Type Culture Collection (ATCC, Manassas, VA).
5. Complete RPMI culture media: 89% (v/v) RPMI with Glutamax (GIBCO-BRL, Carlsbad, CA), 10% (v/v) fetal bovine serum (FBS), 1% (v/v) PSA (GIBCO-BRL).

6. Fresh cadaver or fetal eyes (Anatomic Gift Foundation or Lions Eye Bank, Portland, OR).

2.5. Molecular Verification

1. Trizol (Sigma, St. Louis, MO).
2. Chloroform.
3. Isopropanol.
4. 75% ethanol.
5. Superscript RT Kit (Invitrogen, Carlsbad, CA) contains 10X buffer and dithiothreitol (DTT) (10 m*M*).
6. RedTaq DNA polymerase (Sigma) contains 10X buffer with 1.5 m*M* $MgCl_2$.
7. Deoxynucleotide 5′-triphosphates (dNTPs).
8. Nuclease-free water.

3. Methods

3.1. Three-Plasmid Cotransfection

1. Prepare a 75-cm^2 flask by coating the inside bottom of the flask with 3 mL of attachment factor, place in an incubator for 30 min (*see* **Note 1**).
2. Remove the attachment factor and seed 1×10^5 293T cells to the flask, add 7 mL of DMEM media, and incubate at 37°C with 5% CO_2 (*see* **Note 2**).
3. Grow to 50–80% confluence with media changes every two days (*see* **Note 3**).
4. Prepare a 1.5-mL Eppendorf with the following; 15 μg of lentiviral plasmid (plasmid backbone with gene of interest), 15 μg of ΔR8.9 plasmid, 3 μg of VSV-G plasmid (*see* **Note 4**). Bring the volume to 500 μL with HBS buffer and add 32 μL of 2 *M* $CaCl_2$. Mix thoroughly and incubate at room temperature for 45 min (*see* **Note 5**).
5. Remove media from the 293T cells and pipet the three-plasmid mix onto the monolayer, incubate 15 min at room temperature, gently rock flask to redistribute the mixture, and incubate an additional 15 min (*see* **Note 6**).
6. Carefully add 7 mL of DMEM culture media to the cells/DNA and incubate at 37°C for 12–24 h.
7. Carefully remove the media-DNA mix and replace with 7 mL DMEM containing 10 m*M* sodium butyrate and 20 m*M* HEPES. Incubate at 37°C for 24 h (*see* **Note 7**).
8. Remove and discard the sodium butyrate containing media. Add 7 mL fresh DMEM containing 20 m*M* HEPES to the cells, incubate for 24 h, and collect media. Repeat this incubation and collection for 3 d.

3.2. Lentivirus Collection

1. Pool the collected media (~21 mL) and filter through a 0.2-μm filter into a fresh Oak Ridge centrifuge tube (*see* **Note 8**).
2. Centrifuge the virus containing media at 40,000*g* (in a Beckman, Palo Alto, CA, LM-60 ultracentrifuge or equivalent) for 2.5 h.

3. Carefully decant the media from the virus pellet and resuspend in 1 mL of PBS. Transfer to a fresh 1.5-mL Eppendorf and store at –80°C (*see* **Note 9**).

3.3. Viral Quantification

Use the recommended manufacturer's protocol for use of the p21 GAG antigen ELISA kit (*see* **Note 10**).

3.4. Cell Culture and Viral Transduction

3.4.1. Primary Culture of RPE

1. Obtain fresh human cadaver eyes (*see* **Note 11**).
2. Section the eyes along the corneal-scleral edge approximately 2–3 mm posterior to the limbus.
3. Remove the vitreous body and peel away the neural retina using sterile fine forceps.
4. Place 2% dispase in PBS (Irvine Scientific, CA) into the eyecup and incubate at 37°C, 5% CO_2 for 50 min.
5. Remove Bruch's membrane and attached RPE cells by scraping the inside of the eyecup using a sterile inoculating loop.
6. Cut the scraped tissue into small pieces and collect by brief centrifugation (*see* **Note 12**).
7. Plate the collected cells in laminin-coated 6-well tissue culture plates with 6 mL of DMEM per well (*see* **Note 13**).
8. Incubate at 37°C in 5% CO_2, until the RPE cells become confluent (*see* **Note 14**).
9. Split cells once confluent into 6-well or 12-well plates. Use the cells for transduction assays prior to the fifth passage (*see* **Note 15**).
10. Remove media and add 1, 10, and 100 μL of resuspended viral preparation into different wells to test varying dilutions of virus for infection activity. Incubate without media for 20 min at 37°C. Add 1–2 mL of media and incubate for 48 h. Change media if necessary.
11. After 48 h, view plate under an inverted microscope capable of visualizing enhanced green fluorescent protein (EGFP) to confirm expression of the GFP from the reporter gene (*see* **Note 16**).

3.4.2. Culture of Retinoblastoma Cell Lines WERI and Y-79

Retinoblastoma (Rb) is a malignant intraocular tumor that occurs in children as a result of a homozygous loss of expression of the Rb tumor suppressor gene within developing cells of retinal origin ***(30)***. WERI and Y-79 are human retinoblastoma-derived continuous cell lines ***(31,32)*** that express genes that are normally exclusively expressed within photoreceptors ***(33)***. The Y-79 cell line expresses mainly rod-specific genes ***(34)***, whereas the WERI cells, although not Y-79 cells, express the red and green cone pigment genes ***(35)***. The WERI cells also express the human blue cone pigment gene ***(36)***, implying that this Rb

cell line may be derived from a cone photoreceptor precursor. Identification and characterization of rod cell-specific and cone cell-specific *cis*-acting elements utilizing nuclear protein extracts from Y-79 and WERI cells ***(35–37)*** indicate that these Rb cell lines are valuable biological tools for testing the efficacy of photoreceptor-specific promoters for retinal gene therapy.

1. Rapidly thaw frozen WERI and/or Y-79 cells in a 37°C water bath.
2. Transfer the cells to a 15-mL Falcon tube and add 3 to 5 mL RPMI media with 10% FCS, supplemented with 1% PSA.
3. Collect cells by gentle centrifugation at 125*g* for 3 min.
4. Discard media and freezing solution mix and resuspend the cells with RPMI complete media.
5. Grow cells in suspension in a 25-cm^2 tissue culture flask (*see* **Note 17**).
6. Culture cells at 37°C with 5% CO_2 to a maximum density of 6×10^6 before subdividing these cells into fresh media to a density of 1×10^4 (*see* **Note 18**). Cells are routinely subdivided twice a week to maintain the appropriate density.
7. If cells are not to be used immediately, they can be frozen in complete RPMI media containing 5% (v/v) dimethyl sulfoxide (DMSO) and stored in liquid nitrogen (*see* **Note 19**).Collect 1×10^6 cells by centrifugation (125*g* for 3 min) per transduction assay. Remove the media and resuspend the cell pellet in 0.5 to 1 mL of complete media containing viral supernatant (*see* **Note 20**) and transfer to a single well of a 6- or 12-well tissue culture plate.
8. Culture for 1 h at 37°C, 5% CO_2, then transfer cell/viral suspension to a 25-cm^2 flask, add up to 50 mL of fresh complete RPMI media, and culture for 1 wk.
9. Collect cells by centrifugation (*see* **Note 21**) and analyze for the percentage of cells expressing the reporter gene of interest and quantification of the transcription efficiency of the promoter of interest (*see* **Note 22**).

3.4.1. Ex Vivo Culture of Retinal Tissue

1. Obtain fresh human cadaver eyes or eyes obtained at the time of enucleation.
2. Section the eyes along the corneal-scleral edge approx 2–3 mm posterior to the limbus; remove the anterior portion of the eye (lens, iris, cornea).
3. Carefully remove and discard the vitreous body. Using a sterile scalpel, cut the sclera with attached choroid and retina into 0.5-mm^2 squares.
4. Place each dissected tissue square in one well of a 24-well tissue culture plate with the sclera side down and the retina facing up.
5. Add 1 mL of complete RPMI culture media and incubate overnight at 37°C, 5% CO_2 (*see* **Note 23**).
6. Remove the media and immediately place the desired amount of viral vector, resuspended in PBS or culture media, onto the retinal tissue. Incubate the tissue for 20 min at 37°C, 5% CO_2 (*see* **Note 24**).
7. Add 1 mL of RPMI media, supplemented with extra FCS (*see* **Note 23**), to the tissue and continue to incubate at 37°C, 5% CO_2, for no longer than 5–7 d. Change the media every 24 h.

8. Tissue samples may be processed by histology or molecular means in order to analyze for reporter gene expression.
9. Frozen sections can be used to examine histologically which cell types have been transduced. Unstained frozen sections can be readily used to visualize fluorescent proteins.
10. Retinal photoreceptors can by peeled away and separated from the RPE and the choroidal tissue, mRNA can be isolated (*see* **Subheading 3.5.**) separately for each tissue type in order to determine the presence of viral-mediated gene expression.

3.5. Molecular Verification

1. Add 1 mL of Trizol per 50–100 mg of tissue/cells and pipet up and down. Transfer the solution to a sterile 1.5-mL Eppendorf tube.
2. Follow manufacturer's protocol to isolate total RNA (Trizol Reagent, GIBCO-BRL).
3. Perform a standard RT-PCR using oligo (dT) primer for the RT and nested primers specific to EGFP or the gene interest for the PCR. Use a housekeeping gene such as β-actin as a positive control.

4. Notes

1. Attachment factor (AF) is not necessary for the seeding of 293T cells. However, cells are more liable to slough off during transfection and subsequent media collection if AF is not utilized.
2. 1×10^5 cells have consistently yielded appropriate cell populations for transfection within a 48 h time period after seeding.
3. A 50–80% confluence yields the highest transfection. Higher or lower cell populations tend to lower significantly the transfection efficiency.
4. Plasmids should be isolated using an endotoxin free Maxiprep Kit (Qiagen, Valencia, CA). Higher quality plasmid prep is achieved if the vector is propagated in *E. coli* strain DH5α (GIBCO-BRL). Other *E. coli* strains can release large quantities of carbohydrates upon cell lysis and also have high levels of endonuclease activity, resulting in lower quality and lower yields of DNA.
5. Invert tube once every 10–12 min during the incubation stage. A fine hazy precipitate is consistently observed within 45 min using this method.
6. It is essential to redistribute the mixture carefully since over handling at this stage can easily detach the cell monolayer and decrease the transfection rate.
7. Sodium butyrate is not crucial for the transfection but has been shown to enhance foreign DNA expression rates and increases the stability of transfected cells ***(38)***.
8. This filtration step separates cell debris from viral particles contained within the media. This step permits a purer viral pellet after ultracentrifugation.
9. During this step it is often impossible to see a viral pellet, so it is a good idea to mark the outside of the tube and remove the media carefully, either by

pouring or by pipetting. Keep in mind the position of the pellet. During resuspension, it is important to thoroughly pipet up and down to create a homogeneous viral mix.

10. Any standard capsid antigen ELISA kit can be used to quantify the amount of viral particles isolated. This has been a reliable and cost effective means of detecting purified virus.
11. Primary explants of RPE cells are easier to culture from fetal eyes or from eyes obtained from cadavers no greater than 36 h after death.
12. The RPE scrapings can be cut into small pieces in a Petri dish using a sterile scalpel or by repeatedly pipetting the tissue in a 1.5-mL Eppendorf tube through a 1-mL pipet. It may be necessary to add 1 mL of DMEM media to facilitate pipetting. Centrifuge at 1000*g* for 3 min in a benchtop centrifuge.
13. The initial culture media can be supplemented with 15–20% FBS instead of 10%. An initial high FBS content seems to give a better success rate of establishing RPE growth (Stout, J. T., unpublished data). After the first cell split, 10% FBS DMEM can be used.
14. The media can be changed every third day or when a color change (from pink to yellow) occurs.
15. If the RPE cells are not to be used immediately, they can be frozen down in DMEM media containing 10% dimethyl sulfoxide.
16. The time required to observe fluorescence is cell dependent, it takes 48 h in cultured RPE, microvascular vein endothelial (MVEC), and cornea epithelial cells (CECs), while trabecular meshwork cells (TMCs) can take upwards to 5 to 7 d for maximum expression (unpublished data, Stout, J. T.).
17. Both the Y-79 and WERI cell lines have the appearance of free-floating grapelike clusters when grown in suspension. Fill the flask with approximately 50 mL of complete RPMI media and culture with the flask standing upright (the capped end of the flask facing up).
18. Cells were routinely subdivided twice a week to maintain the appropriate density within a 25-cm^2 flask. If a large-scale preparation of cells is required, cells can be cultured in 75-cm^2 or larger flasks.
19. Frozen stocks were prepared by centrifuging approx 3×10^6 cells at 125*g* for 3 min; the pellet was then resuspended in 0.5 mL of complete RPMI culture medium. To this resuspension was added, a drop at a time with tapping to mix the cells/media, 0.5 mL of culture media containing 10% (v/v) DMSO. The resuspended cells were first acclimatized at –20°C for 2 h, then at –70°C for 2 h, before placing in liquid nitrogen for long-term storage.
20. Cells can be resuspended gently by repeatedly passing through a small bore needle or pipet tip.
21. After centrifugation of the cells, discard the media in 10% bleach to destroy any remaining viral particles.
22. Various reporter gene assays exist, such as β-galactosidase, chloramphenicol acetyl transferase (CAT), luciferase, or EGFP gene. The number of cells expressing EGFP can be determined either by counting green cells visualized through

an inverted UV microscope or by fluorescent-activated cell sorting (FACS). The level of reporter gene activity can be measured by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) or by using the luciferase reporter assay system ***(34)***.

23. Supplement the RPMI media with 20% FCS and add enough media to cover completely the tissue.
24. Incubating for longer time periods will dry out the tissue; 20 min is the minimum time required to obtain lentiviral transduction (Stout, J. T., unpublished data).

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References

1. www.sph.uth.tmc.edu/Retnet/disease.htm.
2. Bird, A. C. (1995) Retinal photoreceptor dystrophies LI. Edward Jackson Memorial Lecture. *Am. J. Ophthalmol.* **119,** 543–562.
3. www.retina-international.com/sci-news/rhout.htm.
4. www.retina-international.com/sci-news/retipig.htm.
5. Farrar, G. J., Kenna, P. F., and Humphries, P. (2002) On the genetics of retinitis pigmentosa and on mutation-independent approaches to therapeutic intervention. *EMBO J.* **21,** 857–864.
6. Hao, W. and Fong, H. K. W. (1999) The endogenous chromophore of retinal G protein-coupled receptor opsin from the pigment epithelium. *J. Biol. Chem.* **274,** 6085–6090.
7. Chen, P., Hao, W., Rife, L., et al. (2001) A photic visual cycle of rhodopsin regeneration is dependent on RGR. *Nat. Genet.* **28,** 256–260.
8. Morimura, H., Saindelle-Ribeaudeau, F., Berson, E. L., and Dryja, T. P. (1999) Mutations in RGR, encoding a light-sensitive opsin homologue, in patients with retinitis pigmentosa. *Nat. Genet.* **23,** 393–394.
9. Gu, S. M., Thompson, D. A., Srikumari, C. R., et al. (1997) Mutations in RPE65 cause autosomal recessive childhood-onset severe retinal dystrophy. *Nat. Genet.* **17,** 194–197.
10. Morimura, H., Fishman, G. A., Grover, S. A., Fulton, A. B., Berson, E. L., and Dryja, T. P. (1998) Mutations in the RPE65 gene in patients with autosomal recessive retinitis pigmentosa or leber congenital amaurosis. *Proc. Natl. Acad. Sci. USA* **95,** 3088–3093.
11. Maw, M. A., Kennedy, B., Knight, A., et al. (1997) Mutation of the gene encoding cellular retinaldehyde-binding protein in autosomal recessive retinitis pigmentosa. *Nat. Genet.* **17,** 198–200.
12. Katsanis, N., Shroyer, N. F., Lewis, R. A., et al. (2001) Fundus albipunctatus and retinitis punctata albescens in a pedigree with an R150Q mutation in RLBP1. *Clin. Genet.* **59,** 424–429.

13. Majid, M. A., Smith, V. A., Easty, D. L., Baker, A. H., and Newby, A. C. (2002) Adenovirus mediated gene delivery of tissue inhibitor of metalloproteinases-3 induces death in retinal pigment epithelial cells. *Br. J. Ophthalmol*. **86,** 97–101.
14. Zhu, X., Hadhazy, M., Groh, M. E., Wheeler, M. T., Wollmann, R., and McNally, E. M. (2001) Overexpression of gamma-sarcoglycan induces severe muscular dystrophy. Implications for the regulation of Sarcoglycan assembly. *J. Biol. Chem*. **276,** 21,785–21,790.
15. Miller, D. G., Adam, M. A., and Miller, A. D. (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol*. **10,** 4239–4242.
16. Roe, T., Reynolds, T. C., Yu, G., and Brown, P. O. (1993) Integration of murine leukemia virus DNA depends on mitosis. *EMBO J*. **12,** 2099–2108.
17. Bennett, J., Wilson, J., Sun, D., Forbes, B., and Maguire, A. (1994) Adenovirus vector-mediated in vivo gene transfer into adult murine retina. *Invest. Ophthalmol. Vis. Sci*. **35,** 2535–2542.
18. Li, T., Adamian, M., Roof, D. J., et al. (1994) In vivo transfer of a reporter gene to the retina mediated by an adenoviral vector. *Invest. Ophthalmol. Vis. Sci*. **35,** 2543–2549.
19. Reichel, M. B., Ali, R. R., Thrasher, A. J., Hunt, D. M., Bhattacharya, S. S., and Baker, D. (1998) Immune responses limit adenovirally mediated gene expression in the adult mouse eye. *Gene Ther.* **5,** 1038–1046.
20. Ali, R. R., Reichel, M. B., Thrasher, A. J., et al. (1996) Gene transfer into the mouse retina mediated by an adeno-associated viral vector. *Hum. Mol. Genet*. **5,** 591–594.
21. Miyoshi, H., Takahashi, M., Gage, F. H., Verma, I. M. (1997) Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. *Proc. Natl. Acad. Sci. USA* **94,** 10,319–10,323.
22. Pittler, S. J. and Baehr, W. (1991) Identification of a nonsense mutation in the rod photoreceptor cGMP phosphodiesterase beta-subunit gene of the *rd* mouse. *Proc. Natl. Acad. Sci. USA* **88,** 8322–8326.
23. Lolley, R. N. and Lee, R. H. (1990) Cyclic GMP and photoreceptor function. *FASEB J*. **4,** 3001–3008.
24. Danciger, M., Blaney, J., Gao, Y. Q., et al. (1995) Mutations in the PDE6B gene in autosomal recessive retinitis pigmentosa. *Genomics* **30,** 1–7.
25. McLaughlin, M. E., Ehrhart, T. L., Berson, E. L., and Dryja, T. P. (1995) Mutation spectrum of the gene encoding the beta subunit of rod phosphodiesterase among patients with autosomal recessive retinitis pigmentosa. *Proc. Natl. Acad. Sci. USA* **92,** 3249–3253.
26. Takahashi, M., Miyoshi, H., Verma, I. M., and Gage, F. H. (1999) Rescue from photoreceptor degeneration in the rd mouse by human immunodeficiency virus vector-mediated gene transfer. *J. Virol*. **73,** 7812–7816.
27. Gouras, P., Kjeldbye, H., and Zack D. J. (1994) Reporter gene expression in cones in transgenic mice carrying bovine rhodopsin promoter/lacZ transgenes. *Vis. Neurosci*. **11,** 1227–1231.

28. Kennedy, B. N., Goldflam, S., Chang, M. A., et al. (1998) Transcriptional regulation of cellular retinaldehyde-binding protein in the retinal pigment epithelium. A role for the photoreceptor consensus element. *J. Biol. Chem.* **273,** 5591–5598.
29. Boatright, J. H., Nickerson, J. M., and Borst, D. E. (2000) Site-specific DNA hypomethylation permits expression of the IRBP gene. *Brain Res.* **887,** 211–221.
30. Lee, W. H., Bookstein, R., Hong, F., Young, L. J., Shew, J. Y., and Lee, E. Y. (1987) Human retinoblastoma susceptibility gene: cloning, identification and sequence. *Science* **235,** 1394–1399.
31. McFall, R. C., Sery, T. W., and Makadon M. (1977) Characterization of a new continuous cell line derived from a human retinoblastoma. *Cancer Res.* **37,** 1003–1010.
32. Reid, T. W., Albert, D. M., Rabson, A. S., et al. (1974) Characteristics of an established cell line of retinoblastoma. *J. Natl. Cancer Inst.* **53,** 347–360.
33. Bogenmann, E., Lochrie, M. A., and Simon, M. I. (1988) Cone cell-specific genes expressed in retinoblastoma. *Science* **240,** 76–78.
34. Di Polo, A. and Farber, D. B. (1995) Rod photoreceptor-specific gene expression in human retinoblastoma cells. *Proc. Natl. Acad. Sci. USA* **92,** 4016–4020.
35. Shaaban, S. A. and Deeb, S. S. (1998) Functional analysis of the promoters of the human red and green visual pigment genes. *Invest. Ophthalmol. Vis. Sci.* **39,** 885–896.
36. Appukuttan, B., Dulai, K. S., and Hunt, D. M. (1996) Isolation and characterization of protein binding sites in the 5′ flanking region of blue cone pigment (BCP) genes. *Invest. Ophthalmol. Vis. Sci.* **37,** S337.
37. Borst, D. E., Boatright, J. H., Jing-Sheng, S., et al. (2001) Structural characterization and comparison of promoter activity of mouse and bovine interphotoreceptor retinoid-binding protein (IRBP) gene 5′ flanking regions in WERI, Y79, chick retina cell, and transgenic mice. *Curr. Eye Res.* **23,** 20–32.
38. Gorman, C. M., Howard, B. H., and Reeves, R. (1983) Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res.* **11,** 7631–7648.

18

HIV-2 Vectors

Suresh K. Arya, Jean E. Cho, and Suzie H. Chang

1. Introduction

Lentivirus vectors are expected to become excellent tools for gene transfer in gene therapy and functional genomics and for engineering transgenic animals. When fully developed, their use will cover a whole gamut of endeavors, from discovery to therapeutics. Because they integrate into the host cell genome and become a permanent member of the genetic makeup of the recipient cell, retroviruses are well suited for stable expression of the transgenes. This property is shared by all retroviruses, including lentiviruses ***(1)***. Apart from the poor suitability of the conventional retroviral vectors for human gene therapy, what brought the prospects of lentiviral vectors to the forefront was their ability to cause gene transfer into nondividing, quiescent cells as well as dividing cells ***(2)***. This attribute will become increasingly important as more knowledge is gained about the biology of stem cells and a more optimized technology is developed for transplantation of genetically modified cells.

The unique ability of lentiviruses to infect nondividing cells stems from the special features of their genetic structure and biology. In addition to the usual structural genes possessed by all retroviruses, lentiviruses contain a number of regulatory and accessory genes. They also contain specific response elements that are targeted by these viral genes and by cellular factors. For example, they contain the transcriptional activator gene, termed Tat, and the corresponding *cis*-acting response element, termed transcriptional activation response (TAR) element, forming a transcriptional regulatory loop. These viruses also possess a posttranscriptional Rev : RRE (Rev responsive element) loop that regulates RNA processing and transport. Moreover, they contain more than one gene with a nuclear localization signal that facilitates nuclear import of the preintegration

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complex in nondividing cells. These genes include Vpr and Vpx, which are thought to be the major instruments of nuclear import ***(3)***.

Another feature that sets lentiviruses apart from other retroviruses is the phenomenon of transcriptional silencing. Conventional retroviruses are known to undergo silencing, specially during early development. This is specially relevant to generating transgenic animals. There has been no evidence so far that the lentiviruses undergo silencing. Recent successful use of lentiviral vectors to create transgenic rodents supports the idea that the lentiviral vectors may be immune to developmental silencing and thus could be excellent tools for creating transgenic animals ***(4,5)***.

We think that vectors derived from human immunodeficiency virus type 2 (HIV-2) may have certain advantages even over those vectors derived from HIV type 1 (HIV-1). HIV-2 is generally less pathogenic than HIV-1, and thus, it may be safer to handle in the design and production stages and presumably also in clinical practice.* The desirable karyophilic nuclear import function of HIV-2 is encoded by the single function *vpx* gene ***(3)***. The same function in HIV-1 is encoded by the *vpr* gene, which also causes cell cycle arrest. HIV-2 vectors may be simpler to design as the cell cycle arrest and nuclear import functions are already segregated. For gene therapy of HIV-1 infection, HIV-2 vectors will be better in being less likely to generate recombinants with the resident HIV-1 genome because of their sequence divergence. Moreover, HIV-2 may itself down regulate HIV-1 ***(6,7)***.

1.1. Strategy and Design

Lentiviruses are more complex than the conventional retroviruses in having several regulatory loops. While this adds to the complexity for understanding their biology, it also provides opportunities for exploitation. Thus, a number of options exist, depending on the nature of the desired gene transfer and attendant safety concerns. The determinants of the two requirements—efficiency and safety—may not always be coincident. There are two aspects to efficiency: (1) efficiency of the delivery of the transgene to the target cells and (2) level of expression of the transgene therein. Similarly, safety needs to be considered at two levels: (1) safety at the time of vector production and (2) safety in the patient, including adverse immune reaction, as well as bystander consequence for the hospital staff and family members.

1.2. Split Genome and Subgenomic Vector Design

The most efficient vehicle for gene transfer would seem to be the virus itself. To remain within the constraints of genetic space, replacing nonessential

*The HIV-2 vectors used in our laboratory are derived from nonpathogenic strain ST.

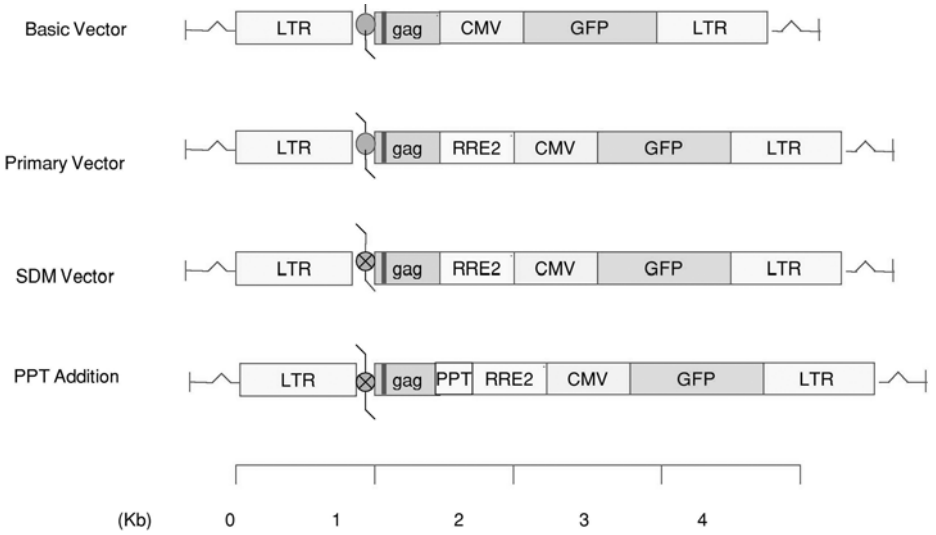

Fig. 1. Path to prototypic HIV-2 transducing vector. These vectors contain about 400 nucleotides of the gag gene with substitution of the Gag ATG by TAG (solid line denotes termination of the open reading frame). The RRE element contained in these vectors is about 450 nucleotides. Circle denotes the splice donor site and cross within it indicates that it has been mutated. PPT is the central polypurine track.

accessory genes with the transgene in the lentivirus genome would seem to be the strategy to follow. However, lentiviruses are not innocuous agents (safety concerns rule out such an approach). Thus, there has evolved the strategy of split genomes, in which gene functions are placed on separate plasmids. Two major splits of the viral genome have been affected to create two vector plasmids, called here transducing vector and packaging vector.

1.3. Transducing Vectors

The transducing vector contains the transgene and the associated *cis*-acting elements to accomplish the following tasks: to transcribe vector RNA in a packagable form in the packaging cell line, to convert the RNA into the DNA form in the target cell, to integrate into the host genome, and to express the transgene RNA. Thus, it must contain promoter-enhancer elements for vector RNA synthesis, a packaging signal for RNA encapsidation into vector particles, and elements for reverse transcription, nuclear import, and integration into the host genome. It may also contain additional internal promoter-enhancer elements for transgene expression (**Fig. 1**).

1.3.1. Promoters

Two configurations of the promoter are possible. The basic vector contains the 5′-LTR (long terminal repeat) that provides the native promoter for vector expression in the packaging cell line and for reverse transcription and integration in the target cell. This promoter can also direct the expression of the transgene in the target cells. However, the LTR-directed transcription needs Tat to overcome transcriptional pausing. The LTR-directed vector thus will express efficiently only in cells that can provide Tat in *trans*. Such a vector theoretically could be selective for HIV-infected cells. Alternatively, the vector could be designed to contain Tat gene as a part of the transcriptional unit, perhaps separated by an internal ribosomal entry site (IRES) element ***(8)***.

The more common promoter-enhancer configuration consists of the 5′ LTR for vector RNA transcription in the packaging cell line (with Tat being provided in trans by the packaging vector) and an internal promoter to drive the expression of the transgene in the target cell. This configuration allows the flexibility of introducing tissue specific internal promoters to express the transgene and thus achieve targeting at the intracellular level.

1.3.2. Packaging Signal

Packaging signals of HIV-2 and HIV-1 have been mapped in some detail ***(9–13)***. These signals in HIV-2 and HIV-1 are generally similar, though the packaging signal of HIV-2 is more extended and complex. In an apparent contrast to the conventional retroviruses, sequence elements both upstream and downstream of the major spliced donor site in the leader sequence of HIV-1 and HIV-2 appear to be required for optimal encapsidation of the vector RNA. Thus, both intronic and exonic sequences participate in encapsidation.

1.3.3. Rev Response Element

In normal virus replication, the unspliced viral genomic RNA is restricted to the nucleus. Its export to the cytoplasm for encapsidation is achieved by Rev. Rev binds to RRE and promotes RNA export ***(14)***. In addition, viral RNA contains several *cis*-acting repressor sequences/inhibitory sequences (CRS/INS), and Rev function overcomes this repression ***(15)***. Thus, RRE must be included in the design of the transducing vector, and Rev must be provided in *trans* by the packaging vector.

1.3.4. Sequence Elements for Reverse Transcription and Integration

In addition to the *cis*-acting promoter-enhancer sequences, the 5′ and 3′-LTRs provide specific sequences for reverse transcription and terminus sequences for integration. One must also ensure that the primer binding site for

Table 1
Comparison of HIV-2 Vectors With and Without Intact Splice Donor

Tranducing Vector[a]	Packaging Vector (core)	Envelope	Titer (TU/mL) (unconcentrated)
HIV-2(SD/RR/CM-GFP)	HIV-2	VSV-G	$4.2 \pm 2.0 \times 10^4$
HIV-2(SDM/RR/CM-GFP)	HIV-2	VSV-G	$3.2 \pm 1.3 \times 10^5$
HIV-2(SDM/RR/_GFP)	HIV-2	VSV-G	$\pm 10^4$
BACKBONE VECTOR	HIV-2	VSV-G	<<

[a]SD, intact splice donor; SDM, mutated splice donor; _, no internal promoter

the minus strand synthesis and 3′-polypurine track for the plus strand synthesis are provided to generate proviral DNA. Inclusion of the central polypurine track reportedly enhances transduction efficiency somewhat ***(16)***.

1.3.5. Splice Sites

In most lentiviral vector design, the major splice donor site in the leader sequence is retained, and a splice acceptor site is provided downstream. This design models native viral RNA in which a fraction of the primary transcripts remains unspliced for packaging into progeny virus particles and in which the other fraction is spliced to function as mRNA. By itself, this design would seem to be inappropriate because the aim should be to package all RNA. However, this design has an advantage in safety. The splice donor and acceptor sites flank the packaging signal that will be spliced out during RNA processing. This result will reduce the fraction of packagable RNA in the target cell. Unfortunately, it will also occur in the packaging cell line, thus reducing packagable RNA. While this may be detrimental for vector production, it also reduces vector mobilization. We have found that for HIV-2 vectors, specifically from nonpathogenic HIV-2(ST), inactivating mutation of the splice donor greatly improves vector titer **(Table 1)** ***(17)***.

1.3.6. SIN Vectors

The self-inactivating (SIN) vectors are designed to express vector RNA in the packaging cell line but be inactive in transcribing RNA from the LTR in the transduced cell ***(1,18)***. This action must occur without impairing transcription from the internal promoter and is accomplished by deleting the promoter-enhancer in the U3 region of the 3′-LTR. This deletion is then copied also into the 5′-LTR upon reverse transcription during provirus synthesis. In addition to curtailing vector packaging in the transduced cell, it also eliminates any promoter interference, as well as activation of cellular genes that may happen

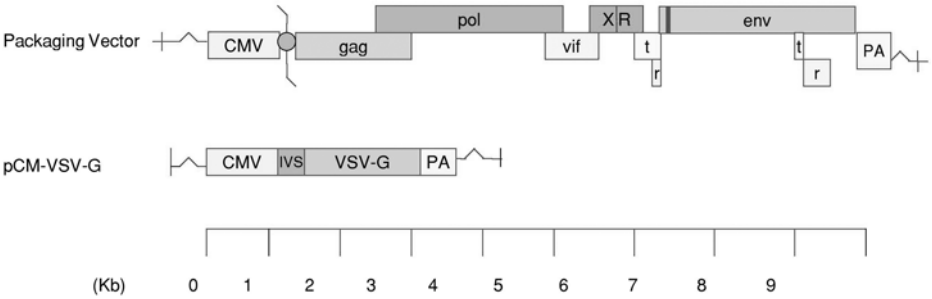

Fig. 2. HIV-2 packaging vector and VSV-G envelope clone. t, *tat*; r, *rev*; solid line in *env* gene denotes truncation.

to be downstream of the provirus integration site, often called insertional mutagenesis. SIN vectors for HIV-2 have not been reported.

1.4. Packaging Vectors

The packaging vectors must provide all the gene products that are needed for particle assembly and package transducing vector RNA, but not its own mRNA **(Fig. 2)**. Thus, packaging vector is composed of whole- or nearly whole-length viral genome with deletion of the packaging signal. Furthermore, to minimize recombination with the transducing vector in the packaging cell line, the 5′-LTR is replaced with a heterologous promoter (for example cytomegalovirus, CMV, promoter) and the 3′-LTR by a heterologous transcriptional terminator (for example, SV40 polyA). Because lentiviruses have restricted host range, the envelope gene in the packaging vector is truncated and an unrelated envelope gene is provided in *trans*. The most commonly used envelope is vesicular stomatitis virus G glycoprotein (VSV-G). The issue of whether some of the accessory genes are beneficial for vector production or transduction is not fully settled. It may depend on the cell type. It appears that the deletion of the accessory genes in HIV-1 packaging vectors is not markedly detrimental, nuclear import function being provided by structural genes or other sequence elements ***(19)***. Multiply-gutted HIV-2 packaging vectors have not been reported. The HIV-2 packaging vectors used in our laboratory have a *nef* deletion.

1.5. Cross-Packaging

Because of the limited sequence homology between HIV-1 and HIV-2, the chance of recombinants will be decreased and safety increased if the vectors could be cross-packaged. Indeed, the HIV-2 transducing vector can be packaged both by the HIV-1 and HIV-2 packaging machinery. Interestingly, the HIV-1 transducing vector in contrast is not as efficiently packaged by HIV-2

Table 2
Cross-Packaging of HIV-1 and HIV-2 Transducing Vectors

Transfer vector	Packaging vector (core)	Envelope	Titer (TU/mL) (unconcentrated)
HIV-2	HIV-2	VSV-G	$1.8 \pm 0.8 \times 10^5$
HIV-2	HIV-1	VSV-G	$2.0 \pm 0.8 \times 10^5$
HIV-1	HIV-2	VSV-G	$2.2 \pm 0.6 \times 10^4$
HIV-1	HIV-1	VSV-G	$1.6 \pm 0.6 \times 10^6$
Untransduced	<<	<<	<<

packaging machinery **(Table 2)**. As with cross-transactivation, there appears to be a nonreciprocal relationship between HIV-1 and HIV-2 packaging. This information is being used to create specific and safer chimeric or hybrid vectors.

2. Materials

The sources listed below are those used by our laboratory. Other comparable and competitive sources can be readily found.

1. Human embryonic kidney transformed 293T cells.
2. Dulbecco's modified Eagle's medium (GIBCO-BRL/Invitrogen, Carlsbad, CA).
3. Fetal bovine serum (GIBCO-BRL/Invitrogen).
4. Antibiotic-antimycotic mixture (GIBCO-BRL/Invitrogen).
5. Phosphate-buffered saline (PBS) (GIBCO-BRL/Invitrogen).
6. Tris-buffered saline (TBS): 50 m*M* Tris-HCl, pH 7.8, 0.13 *M* NaCl, 10 m*M* KCl, 10 m*M* $MgCl_2$.
7. 10X dNTP-SS: 1 m*M* each of dATP, dGTP, dTTP, and dCTP plus 30 m*M* spermine and 3 m*M* spermidine.
8. Fixed-angled 45Ti Rotor, polyallomer tube capacity approx 60 mL (Beckman-Coulter, Fullerton, CA).
9. Swinging Bucket SW41Ti Rotor, polyallomer tube capacity approx 12 mL (Beckman-Coulter).
10. Calcium Phosphate Transfection Kit—Profection Mammalian Transfection System (Promega, Madison, WI).
11. SIV p27 Antigen Capture Kit for HIV-2 p27 determination (Beckman-Coulter).

3. Methods

3.1. Vector Cloning

Standard recombinant procedures are used to create vector clones. HIV-2 provirus plasmid clones to be used as a starting point for construction may

be obtained from laboratories engaged in molecular studies with HIV-2. Most laboratories require a material transfer agreement (MTA) to be signed for exchange of reagents. Lambda phage clones of HIV-2(ST) and HIV-2(ROD) are also available to qualified investigators from the AIDS Research and Reference Program of the U.S. National Institute of Allergy and Infectious Diseases, National Institutes of Health: http://www.aidsreagent.org

3.2. Vector Production

The protocol below is for preparing vectors by transient transfection of monolayers of human embryonic kidney transformed 293T by the calcium phosphate procedure and for cells grown in 175-cm^2 T flasks. Other flasks and dishes can be used and reagents scaled up and down accordingly. All procedures using cells are done in a biosafety hood and cultures are grown in humidified 5% CO_2 incubator (*see* **Note 1**).

1. A day before transfection, plate 1.6–2.0 × 10^6 cells in 20–24 mL medium. Grow them overnight.
2. About 4 h before transfection, replace the spent medium with fresh 20–24 mL medium.
3. Prepare DNA-calcium phosphate precipitate in a tissue culture hood according to the procedure recommended by the supplier of the transfection kit (*see* **Note 2**). For the kit provided by Promega Corporation, aliquot enough water (usually about 850 μL) into a sterile polypropylene tube (e.g., Falcon 17 × 100 mm) so that final DNA-calcium solution will be 1.0 mL. Add 25 μg of plasmid DNA of the transducing vector, 20 μg of plasmid DNA of the packaging vector, and 5 μg of VSV-G plasmid DNA and mix (*see* **Notes 3** and **4**). Add 124 μL of 2 *M* calcium chloride solution and mix.
4. In a separate sterile tube, aliquot 1.0 mL of 2X HBS buffer.
5. To this buffer aliquot, add dropwise and slowly Ca-DNA solution over a period of 3–5 min while gently bubbling air or nitrogen through the solution (*see* **Note 5**). As the addition progresses, the mixture should appear slightly milky (but not granular) because of the formation of the DNA-calcium phosphate precipitate.
6. Let the mixture stand at room temperature for 20 min.
7. Before adding to the cells, resuspend the precipitate by pipetting or gentle vortexing of the mixture. Add the mixture dropwise to the culture flask while gently swirling the flask to cause even distribution of the precipitate. The cultures should be 20–40% confluent at this stage and the precipitate should appear as a fine dust covering the monolayer when examined under the microscope. Return the flask to the incubator avoiding any sudden shaking.
8. Twelve to sixteen hours after transfection, pour off the medium, gently wash the culture once or twice with 16–20 mL PBS and once with 16–20 mL of medium; add 16–20 mL of fresh medium.
9. Incubate the culture for an additional 24–48 h (*see* **Note 6**).

10. Harvest the medium, clarify it of any cell or large cell debris by slow speed centrifugation (3000*g*, 10 min) in a tabletop centrifuge, and filter the medium through a 0.45-μm pore size filter to remove finer debris.

If the medium is to be used directly as a vector for transduction, distribute it into 200–400-μL aliquots and store at –80°C (*see* **Note 7**).

3.3. Vector Concentration

To obtain higher titer vector, VSV-G pseudotyped vectors can be concentrated by ultracentrifugation.

1. Transfer the medium to an appropriate sterile centrifuge tube and centrifuge at 60,000*g* for 100 min at 12°C (*see* **Note 8**).
2. Pour off the supernatant and add TBS buffer equivalent to 1/20th to 1/40th volume of the original medium. For a 20-mL harvest, 0.5 mL to 1.0 mL of buffer is adequate. Suspend the pellet by placing the tube in ice for 30 min with occasional mixing. Pipet the solution up and down several times to achieve suspension.
3. Add 1/10th volume of 10X dNTP-SS solution. Incubate at 37°C for 1–2 h (*see* **Note 9**).
4. Centrifuge the solution again at 60,000*g* for 100 min. Suspend the pellet as before but in PBS containing 4 μg/mL polybrene in a minimum volume or in a volume equivalent of 1/1000th–1/200th volume of the original medium (*see* **Note 10**).

3.4. Vector Titration

This protocol is for green fluorescence protein (GFP) transgene readout titered on 293 T cells. It can be modified to apply to other transgenes for which an assay is available.

1. In a 12-well plate, seed 2–4 × 10^4 cells/well a day before transduction and incubate at 37°C. Plate extra wells to serve as control wells, which do not receive vectors and are for cell counting.
2. Make serial 5- or 10-fold dilutions of the vector into 1.0 mL of medium containing 8 μg/mL polybrene. The serial dilution should cover the range of expected vector titer, usually 10^2 to 10^6. Transfer diluted vectors to cells in individual wells and incubate overnight or for 16–20 h. Also, count cells in duplicate wells to determine the cell number at transduction.
3. Siphon off the medium and incubate with 1.0 mL of fresh medium for 2–3 d.
4. Cultures can be monitored at this stage by fluorescence microscopy with or without fixing. Count several fields for total number of cells and green fluorescent cells (*see* **Note 11**).
5. For more quantitative determination, prepare cultures for flow cytometry. Siphon off medium and wash cells with 1 mL PBS. Detach cells from the plastic surface

by incubating them with 1 mL of 1 m*M* ethylenediaminetetraacetic acid (EDTA) in PBS for 15 min at room temperature. Suspend cells into single-cell suspension by repetitive pipetting. Count an aliquot of the suspension to determine cell numbers. It is sufficient to count only a few representative wells.

6. To fix cells, centrifuge the suspension at low speed. Suspend the pellet in 1 mL of a 2% paraformaldehyde in PBS and place them on ice for 30–60 min. Pellet the cells again and suspend them in PBS containing 2% serum.
7. Analyze for GFP expression by flow cytometry as recommended by the manufactures of the flow cytometer. Determine the percentage of GFP-positive cells (*see* **Note 12**).
8. Calculate the titer of the vector by plotting the data to determine the dilution that gives 50% cells as GFP-positive and compute transduction units from that dilution. Alternatively, transduction units can be computed for several dilutions and the average determined as follows:

   % GFP-positive cells/100 × number of cells at transduction × dilution factor

   This will give the titer in terms of transducing units (TU)/ml. A better assessment of the quality of the vector is obtained when titer is expressed per particle or surrogate marker of the particle–p27 content (TU/ng p27). The p27 antigen can be determined by standard antigen capture assay using a commercially available kit.

3.5. RCR Detection

A convenient and semiquantitative way to detect generation of replication-competent recombinants (RCR) is to perform sequential transduction of fresh target cells.

1. For GFP vectors, add 0.5 to 1.0 mL of undiluted and 10-fold diluted vector in medium with 8 μg/mL polybrene to duplicate cultures of 0.5×10^6 293T cells in a small flask or dish (for example, 25-cm^2 T-flask or 50-mm dish) and incubate overnight at 37°C.
2. Wash culture twice with PBS, once with medium, and incubate in 1 mL of fresh medium for 2 d. The culture may be analyzed for GFP expression by flow cytometry to reevaluate quality of the vector.
3. Harvest the medium, clarify by slow speed centrifugation, filter through 0.45-μm pore size filter, apply it to a fresh culture as before, and analyze the culture for GFP expression. The culture should be negative for GFP-positive cells, except for a possible carryover.
4. Perform at least one more serial passage to rule out artifacts.

An alternative to GFP-expressing cells would be to monitor the culture for the production of p27 core antigen upon serial passage. A more sensitive assay for RCR generation and mobilization for HIV-1-derived vectors has been described ***(20)***. This assay can be adapted to evaluate HIV-2 vectors.

4. Notes

1. Check for the biosafety requirements of your institution.
2. Solutions for transfection can be made in the laboratory also. HEPES-buffered saline (HBS): 50 m*M* N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 7.1, 0.28 *M* NaCl, 1.5 m*M* Na_2HPO_4, pH 7.1.
3. Adjust the concentration of the plasmid DNA to 1–2 mg/mL in TE buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.4) by dilution or by ethanol precipitation and redisolution.
4. It is advisable to produce a "control" vector in which either the packaging vector or the VSV-G plasmid DNA is omitted. This procedure allows evaluation of carryover DNA at transduction. This is particularly important if the supernatant from the transfected cultures is to be used directly as a vector.
5. A convenient way of adding Ca-DNA to HBS buffer is to blow air through the solution with a Pasteur pipet attached to a pipettor and held in one hand while adding drops of the Ca-DNA solution with the other hand.
6. Monitor the cultures. They preferably should not be too confluent and dense before the medium is harvested.
7. For long-term storage, store the vector in a liquid nitrogen vessel if available.
8. For example, in a Beckman 45Ti at 28,000 rpm. Tubes of this rotor hold approx 60 mL medium.
9. This step presumably initiates reverse transcription and makes the vector more efficient in transduction; however, it seems to be based on rather flimsy evidence and can be omitted without much loss in efficiency. If this preincubation is not to be included, the pellet can be suspended in PBS instead of TBS buffer.
10. To obtain concentrated vector, it is better to start with multiple culture flasks, so that the volumes do not become too small to be manageable.
11. The cultures should be subconfluent for proper counting.
12. Fixed cells can be stored in a refrigerator for several days before analysis.

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References

1. Miller, A. D. (1997) Development and application of retroviral vectors, in *Retroviruses* (Coffin, J. M., Huges, S. H., and Varmus, H. E., eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 437–473.
2. Naldini, L., Blomer, U., Gallay, P., et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.

3. Fletcher, T.M., Brichacek, B., Sharova, N., et al. (1996) Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIVsm. *EMBO J.* **15,** 6155–6165.
4. Lois, C., Hong, E. J., Pease, S., Brown, E. J., and Baltimore, D. (2002) Germ line transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295,** 868–872.
5. Pfeifer, A., Ikawa, M., Dayn, Y., and Verma, I. M. (2002) Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc. Natl. Acad. Sci. USA* **99,** 2140–2145.
6. Arya, S. K., Zamani, M., and Gallo, R. C. (1994) HIV-2 inhibits HIV-1. *AIDS Res. Hum. Retroviruses* **10,** 515S.
7. Arya, S. K. and Gallo, R. C. (1996) Human immunodeficiency virus (HIV) type 2 mediated inhibition of HIV type 1: a new approach to gene therapy of HIV infection. *Proc. Natl. Acad. Sci. USA* **93,** 4486–4491.
8. Sadaie, M. R., Zamani, M., Whang, S., Sistron, N., and Arya, S. K. (1998) Towards developing HIV-2 lentivirus-based retroviral vectors for gene therapy: dual gene expression in the context of HIV-2 LTR and Tat. *J. Med. Virol.* **54,** 118–128.
9. Garzino-Demo, A., Gallo, R. C., and Arya, S. K. (1995) Human immunodeficiency virus type 2 (HIV-2): packaging signal and associated negative regulatory element. *Hum. Gene Ther.* **6,** 177–184.
10. McBride, M. S., Schwarts, M. D., and Panganiban, A. T. (1997) Efficient encapsidation of human immunodeficiency virus type 1 vectors and further characterization of cis elements required for encapsidation. *J. Virol.* **71,** 4544–4554.
11. McCann, E. M. and Lever, A. M. (1997) Location of cis-acting signals important for RNA encapsidation in the leader sequence of human immunodeficiency virus type 2. *J. Virol.* **71,** 4133–4137.
12. Poeschla, E., Gilbert, J., Huang, S., Ho, A., and Wong-Staal, F. (1998) Identification of a human immunodeficiency virus type 2 (HIV-2) encapsidation determinant and transduction of nondividing human cells by HIV-2 based lentivirus vectors. *J. Virol.* **72,** 6527–6536.
13. Arya, S. K., Zamani, M., and Kundra, P. (1998) HIV-2 lentivirus vectors for gene transfer: expression and potential for helper virus free packaging. *Hum. Gene Ther.* **9,** 1371–1380.
14. Malim, M. H. and Cullen, B. R. (1993) Rev and the fate of pre-mRNA in the nucleus: implications for the regulation of RNA processing in eukaryotes. *Mol. Cell. Biol.* **13,** 6180–6189.
15. Schneider, R., Campbell, M., Nasioulas, G., Felber, B. K., and Pavlakis, G. N. (1997) Inactivation of the human immunodeficiency virus type 1 inhibitory elements allow Rev-independent expression of Gag and Gag/Protease and particle formation. *J. Virol.* **7,** 4892–4903.
16. Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L, and Charnau, P. (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101,** 173–185.

17. D'Costa, J., Brown, H. M., Kundra, P., Davis-Warren, A., and Arya, S. K. (2001) Human immunodeficiency virus type 2 lentiviral vectors: packaging signal and splice donor in expression and encapsidation. *J. Gen. Virol.* **82,** 425–434.
18. Cui, Y., Iwakuyma, T., and Chang, L. (1999) Contribution of viral splice sites and cis-regulatory elements to lentivirus vector function. *J. Virol.* **73,** 6171–6176.
19. Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L, and Torno, D. (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat. Biotech.* **15,** 871–875.
20. Wu, X., Wakefield, J. K., Hongmei, L., Xiao, H., Kralovics, J. T., and Kapes, J. C. (2000) Development of a novel trans-lentiviral vector that affords predictable safety. *Mol. Ther.* **2,** 47–55.

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SIV Vectors

Alessia Ruggieri, Didier Nègre, and François-Loïc Cosset

1. Introduction

The recovery of vectors that are suitable for an in vivo gene delivery has been a recurrent theme in gene therapy research over the last decade. Several challenging hurdles need to be overcome to reach such a goal. First, is a need for methods that allow the preparation of vectors at high titers and in culture systems with potential for large scale-up need to be optimized. Second, the gene transfer vectors should not be recognized by the host immune system in order to avoid inactivation. Upon delivery into gene therapy recipients, vectors should also be able to circumvent the numerous biological barriers that are likely to limit their diffusion and biodistribution in the target organism. They should, therefore, be able to recognize specifically and to penetrate cells of the gene therapy target tissue. Third, they should be able to replicate and to express a transgene in cells that are either not or only slowly proliferating, a predominant situation in vivo. Last, but not least, they should be accepted by both ethical and regulatory authorities. In this respect, the development of vectors derived from viruses that are not pathogenic to human may be preferred.

Vectors derived from retroviruses offer particularly flexible properties in gene transfer applications given the numerous possible associations of various viral surface glycoproteins (determining cell tropism and interaction with the environment) with different types of viral cores (determining genome replication and integration) ***(1)***. For example, association of the vesicular stomatitis G glycoprotein (VSV-G) with viral cores derived from lentiviruses results in pseudotyped vectors that have broad tropism and can integrate into nonproliferating target cells ***(2)***. At present, it is not clear which type

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of lentivirus vector (derived from either primate or nonprimate lentiviruses) should be better accepted in clinical settings. It is, therefore, essential to design vectors derived from a wide range of lentivirus types and to compare their properties in terms of transduction efficiency and biosafety.

Lentiviral vectors derived from simian immunodeficiency virus (SIV) have now been generated in several laboratories *(1)*. Characterization of these vectors has indicated that they are similar to those derived from human immunodeficiency virus types 1 or 2 (HIV-1, HIV-2) with respect to the insertion of transgenes in nonproliferating cells. However, it is becoming clear that SIV vectors perform better than HIV-1 vectors in simian cells *(3)*; thus they may provide a valid alternative to HIV-1-based vectors, at least in the early phases of the clinical testing of lentivirus vectors. Here, we describe methods aimed to generate high titers and safe lentiviral vectors derived from SIV. We also focus on the importance of varying the glycoproteins harbored by the vector particles in specific applications, in which stability in serum or efficient gene transfer in primary hematopoietic cells is required.

1.1. SIV Proviral Genome: Keys for the Design of SIV-Derived Vectors

The genetic organization of SIVs is similar to that of the HIV-1 and HIV-2, although there are some exceptions *(4,5)*. SIV proviral genomes **(Fig. 1A)** are composed of two identical long terminal repeats (LTRs) flanking the

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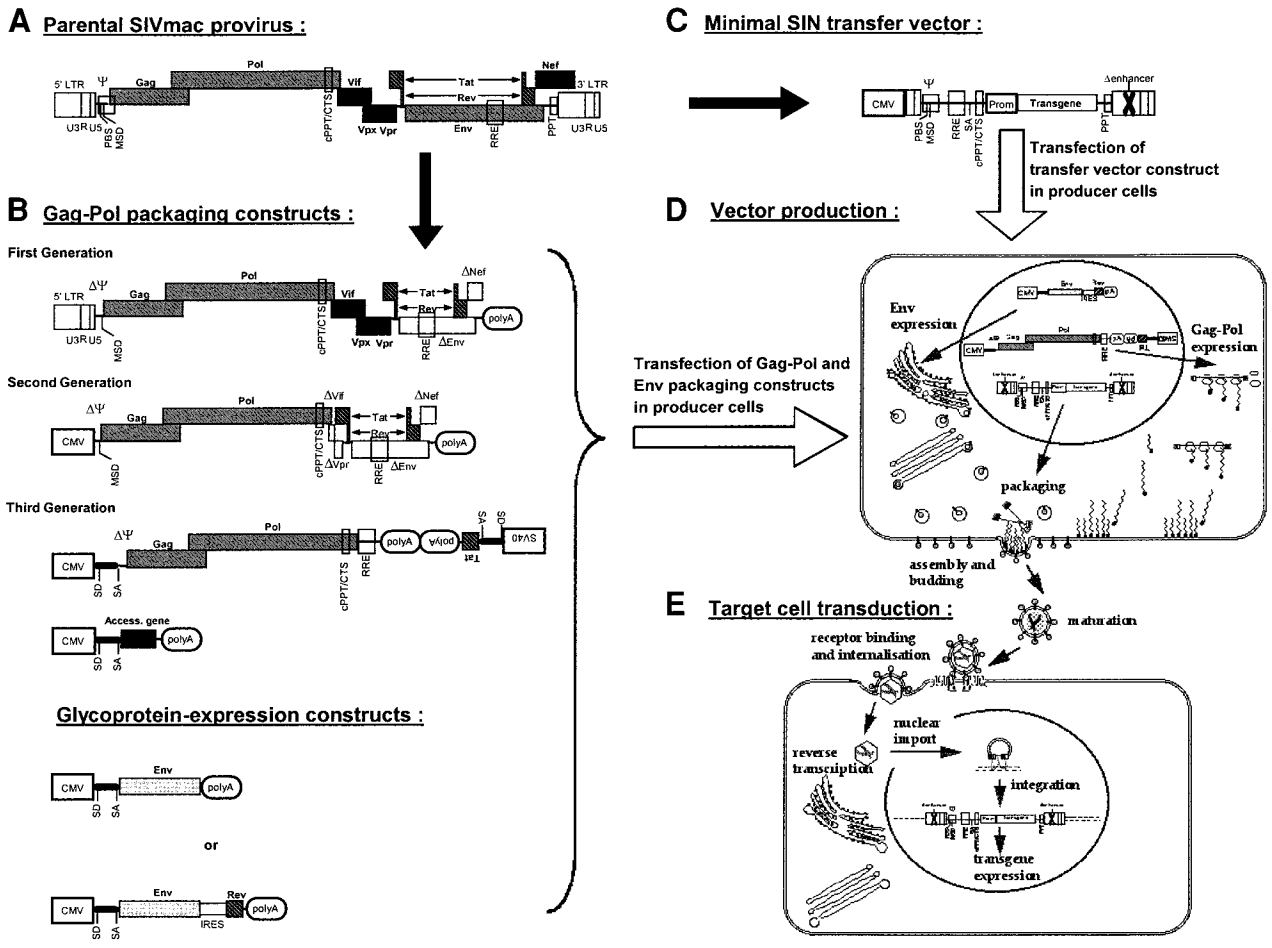
Fig. 1. *(see facing page)* Generation of vectors derived from SIV. The genomes of infectious molecular clones of SIV **(A)** were dismantled to derive constructs encoding the packaging functions **(B)** and constructs carrying the transfer vector **(C)**. Different versions of the Gag-Pol packaging constructs were generated to progressively eliminate the viral sequences unnecessary to formation of vector particles. A similar approach was used to optimize efficient and safe transfer vectors. Only a minimal transfer vector is shown here. The open boxes show the *cis*-acting sequences. The filled boxes represent the viral genes. LTR is long terminal repeat; CMV, human cytomegalovirus early promoter; PBS, primer binding site; MSD, major splice donor site; Ψ, packaging sequence; cPPT/CTS, central polypurine track and central termination sequence; RRE, Rev-responsive element; polyA, polyadenylation site; SD, splice donor site; SA, splice acceptor site; SV40, simian virus 40 early promoter; IRES, internal ribosomal entry signal; Prom, internal promoter. Expression constructs that express the viral glycoproteins (Env) and/or the Rev protein were also designed. Vector particles were produced by cotransfection of plasmids harboring the packaging constructs (Gag-Pol/Tat/Env/Rev) and the transfer vector into 293 cells **(D)**. The supernatants of transfected cells were collected during transient expression and used for target cells transduction **(E)**.


A Parental SIVmac provirus :
5' LTR
Ψ
Gag
Pol
Vif
Tat
Rev
Nef
3' LTR
U3R U5
PBS
MSD
cPPT/CTS
Vpx Vpr
Env
RRE
PPT
B Gag-Pol packaging constructs :
First Generation
ΔNef
ΔΨ
ΔEnv
polyA
Second Generation
ΔVif
ΔVpr
CMV
SD
SA
Third Generation
Access. gene
Glycoprotein-expression constructs :
or
IRES
C Minimal SIN transfer vector :
Δenhancer
Prom
Transgene
Transfection of
transfer vector construct
in producer cells
D Vector production :
Transfection of Gag-Pol and
Env packaging constructs
in producer cells
Env
expression
Gag-Pol
expression
packaging
assembly and
budding
E Target cell transduction :
maturation
receptor binding
and internalisation
nuclear
import
reverse
transcription
integration
transgene
expression

coding regions for the structural genes, *gag*, *pol*, and *env*, which form the viral particles. Like the human lentiviruses, SIVs harbor two regulatory proteins, Tat and Rev, which govern the expression of the viral gene at both transcriptional and posttranscriptional levels. Most SIV isolates also code for virulence factors or accessory proteins, Vpr, Vpx, Vif, and Nef, which are necessary for pathogenicity but not for replication of the viruses in cell culture. The *vpx* gene is found also in HIV-2 but not in HIV-1 isolates, whereas the *vpu* gene, found in HIV-1 but not in HIV-2, is absent from most SIV isolates. Expression of some of these accessory genes in vector producer cells may profoundly impact the performance of the vectors ***(6)***. Like other retroviruses, the SIV LTR is divided into the U3, R, and U5 regions. Signal sequences in U3 regulate the level and the start site of transcription, which begins at the U3/R boundary. The R region contains a *cis*-acting element, the *trans*-acting responsive (TAR) sequence, which is an essential regulatory motif mediating *trans*-activation by the virus-encoded Tat protein. The TAR element forms a stable secondary structure that binds Tat as a complex with cellular factors, allowing elongation of the viral transcript. The R region also contains the polyadenylation site for SIV RNAs. An essential determinant of the viral RNA drives the packaging of two copies of the genomic RNA into the viral particles. This determinant, known as the packaging sequence, is essentially located in the leader region ***(7)***, yet additional sequences located in R-U5 and in the beginning of the gag gene are also likely to be part of this critical motif ***(8–10)***.

Reverse transcription requires the presence of several *cis*-acting elements that are responsible for the initiation of DNA synthesis. They include **(Fig. 1A)** (1) the primer-binding site (PBS), located at the beginning of the leader region between the 5′ LTR and the *gag* gene, which binds a tRNA that primes synthesis of the negative strand of proviral DNA; (2) the central polypurine track (cPPT) and the 3′ PPT regions that are both used to prime the synthesis of the positive strand of the viral DNA; and (3) a conserved uridine-rich sequence located immediately upstream of the PPT in various lentivirus strains ***(11)***. During the reverse transcription process, the 3′ U3 region is used as template for formation of the U3 regions of both the 5′ and 3′ LTRs. Interaction of integrase with both ends of LTRs leads to insertion of the viral DNA into the host cell chromatine.

1.2. Transfer Vectors

Replication-competent vectors derived from SIV have been generated by replacing the nef gene by heterologous sequences ***(12)***. These vectors were shown to be capable of replicating not only their own genome but also the transgenes inserted into their genomes, thus resulting in their efficient dissemination in the host organisms. Although they represent attenuated and

probably not pathogenic forms of the initial viruses because of the deletion of the nef virulence gene, such vectors are nevertheless unsuitable for most gene therapy applications and have in fact been designed as live attenuated vaccine candidates against AIDS ***(13)***.

In general, retroviral vectors can be rendered replication-defective by deleting the critical genes involved in viral replication, i.e., the *gag-pol* and *env* genes. The transgenes and occasionally some regulatory elements (promoters, enhancers, and intron sequences) are inserted into the deleted virus, referred to as the transfer vector **(Fig. 1C)**, which still retains most of the viral *cis*-acting elements, such as LTRs, packaging sequences, and regions involved in reverse transcription (PBS and PPT). Some *cis*-acting sequences overlap with *trans*-acting regions in all retrovirus genomes **(Fig. 1A)**. Consequently, most transfer vectors still contain residual sequences that are derived from genes of the parental virus and whose coding capacities have been inactivated. This is, for example, the case of the packaging sequence that extends into the *gag* gene of the RRE region located in the *env* coding sequence of the cPPT/central termination sequence (CTS) sequences positioned in the *pol* gene and of the PPT sequence found into the *nef* gene **(Fig. 1A)**. Such redundancies between packaging and vector genomes should be avoided whenever possible, or at least minimized, to reduce the possibility of recombination with the packaging genome that carries the *trans*-acting functions. Indeed, despite the deletion of most of the packaging sequence in the latter type of genome, low levels of packaging may still occur ***(14)***.

Based on these considerations, several transfer vector and packaging genomes have recently been derived from different types of SIVs: SIVmac251, ***(3,15–17)*** and SIVagm ***(18,19)***. The minimal configuration required to propagate efficiently these different vectors is shown in **Fig. 1C**. The genomic RNA of the transfer vector can be expressed using wild-type or hybrid 5′ LTRs and required Tat expression in producer cells in *trans* for optimal transcription ***(3,15)***. Vectors may also retain the Rev responsive element (RRE) sequence, although its presence was found dispensable ***(15,16)***, probably owing to the removal of most of the instability sequences ***(20)*** in the vector backbone. Since the packaging sequence is thought to extend into the beginning of the *gag* gene and since packaging has been proposed to occur in *cis* for HIV-2 ***(9,10)***, a virus closely related to SIV, the most efficient SIV-based vectors still contain residual sequences of approx 50 nucleotides derived from this gene ***(15)***. Further deletion of the *gag* gene and of the 3′ end of the leader region have a strong negative influence on vector titers, most likely owing to a too severe disruption of the packaging sequence ***(15)***. Following vector integration in target cells, the transgenes can be efficiently expressed from internal transcription units using strong constitutive promoters that do not require Tat/TAR and

Rev/RRE sequences for optimal expression. This has allowed the construction of vectors that carried large deletions in the 3′ LTR U3 regions, encompassing the binding sites for several transcription factors and the TATA box **(Fig. 1C)**, which had dramatically reduced transcription capacities from the 5′ LTR after a round of reverse transcription and integration in target cells ***(15,16,18)***.

These SIN (self-inactivating) vectors offer several advantages such as (1) preventing possible vector mobilization upon coinfection with a replicant-competent retrovirus (RCR), (2) abolishing the requirement for Tat expression for transgene expression in target cells, and (3) reducing interferences of the SIV LTR with the internal promoter, thereby allowing transgene expression driven by tissue-specific or regulatable internal promoters inserted in the vector backbone ***(6)***. Addition of other *cis*-acting elements in the vectors, which include sequences that increase RNA-export or other posttranscriptional regulation ***(21)*** and transgene expression ***(22)***, have been inserted in SIV-derived vectors and shown to improve vector efficiency in a target cell type-dependent manner ***(6)***. Depending on the particular configurations of the vectors, infectious titers higher than 10^7 infectious particle (i.p.)/mL can be obtained.

1.3. Packaging Genomes

In order to propagate the transfer vector as replication-defective virus particles, it is necessary to derive *trans*-complementing genomes and/or packaging cell lines that provide in *trans* the packaging proteins whose genes have been deleted in the transfer vector **(Fig. 1B)**. The *trans*-complementing genomes, also referred to as the packaging vectors, are designed in such a way that they cannot themselves replicate. This characteristic is usually achieved by removing essential *cis*-acting sequences that pertain to the initial retrovirus, such as the packaging sequence, the PBS and PPT regions, and both LTRs. These modifications of the packaging genomes are in practice sufficient to prevent transfer of SIV genes into transduced cells ***(15)*** and formation of RCRs ***(3)***. Additionally, the packaging proteins are usually encoded by two separate and complementary genomes, a first one for the Gag-Pol proteins and a second one for the viral glycoproteins, which does not necessarily originate from the parental lentivirus **(Fig. 1B)**. This physical separation ensures both high levels of biosafety by minimizing the possibility of recombination and flexibility, and by easily allowing the exchange of glycoproteins of different viral origins.

Widely used packaging vectors of second and third generations are shown in **Fig. 1B**. Since packaging constructs of second generation still contain most of the accessory genes and bear a close resemblance to the parental SIV genome, they are not considered as particularly safe given their high resemblance to the SIV genome. Packaging constructs of the third generation have been designed in such a way that they only contained the genes encoding the structural

proteins *(3,17)*. Since no SIV sequences beyond the stop codon of the *pol* gene are found in these constructs, this theoretically minimizes the chance of forming a viable recombinant virus. Depending on the target cells of interest, SIV vector particles may require some accessory genes. For example, human dendritic cells may only be transduced when the SIV vectors have been generated in the presence of Vpx *(6)*. Such accessory genes should be expressed *in trans* via separate expression vectors rather than by using first generation packaging constructs **(Fig. 1B)**.

1.4. Pseudotyping SIV-Derived Vectors

Protein incorporation on retroviruses is not specific to the homologous viral glycoproteins. More than 40 different host cell-derived proteins have been identified on the exterior of HIV-1 viral particles, including major histocompatibility complex classes I and II (MHC-I and MHC-II) molecules, adhesion molecules, costimulation molecules, and complement control proteins *(23)*. Additionally, many heterologous viral glycoproteins can be incorporated into retrovirus particles and mediate infectivity *(24)*. This process, known as pseudotyping, allows retroviral vectors to transduce a broader range of cells and tissues. Expression of glycoproteins can be achieved from packaging-deficient expression constructs driven by strong constitutive promoters **(Fig. 1B)**. No sequence homology is found between these constructs and the Gag-Pol packaging vector and the transfer vector constructs, thus minimizing the possibility to generate recombinant viruses that have integrated the glycoprotein coding sequences.

There is considerable interest in exploring the properties of lentiviral vectors pseudotyped with alternative viral glycoproteins *(25–32)*. This parameter is likely to modulate the physicochemical properties of the vectors, their interaction with the host immune system, and their host-range. Several studies have indeed shown that the transduction efficiency of target cells is dependent on the type of glycoprotein used to coat retroviral vectors *(31,33–38)*. Additionally, some in vivo gene transfer applications will require vectors that are targeted for specific cell entry and/or gene expression after systemic administration *(39)*. Lentiviral vectors have been mostly generated with VSV-G, as this glycoprotein makes easy the recovery and concentration of pseudotyped vectors *(2,40)*. However, because of the wide distribution of its receptor, a lipid component of the plasma membrane *(41)*, VSV-G pseudotypes may bind to the surface of any cell encountered after inoculation before they reach the target cells. Moreover, VSV-G-pseudotyped vectors are rapidly inactivated by human serum *(31,42)* and this might impose a limitation on the use of VSV-G as a glycoprotein to pseudotype vectors for systemic gene delivery.

Formation of SIV vectors pseudotyped with several glycoproteins have been recently reported, though with different efficiencies *(3,31)*. Infection

assays on TE671 human rhabdomyosarcoma cells indicated that titers higher than 10^5 infectious units (i.u.)/mL are obtained for vectors generated with the glycoproteins (GPs) of VSV, fowl plaque virus (FPV) (*see* **Note 1**), lymphocytic choriomengitis virus (LCMV), and murine leukemia virus-A and -10A1 (MLV-A and MLV-10A1). In contrast, vectors generated with the GPs of Ebola V (EboV) had lower titers, less than 10^4 i.u./mL. SIV vectors generated with the GPs of gibbon ape leukemia virus (GALV) and RD114 have intermediate titers, between 10^4 and 5×10^4 i.u./mL. The infectious titers obtained with SIV vectors generated with the GPs of GALV and RD114 are surprisingly low in comparison to those achieved with MLV vectors pseudotyped with the same glycoproteins ***(38,43,44)***. Further studies have indicated a defect at the level of GP incorporation on the lentiviral cores ***(25,30–32)***. Such a block at the level of assembly of these GPs on viral particles could be overcome by introducing modifications of the cytoplasmic tails. Indeed, replacement of the cytoplasmic tail of RD114 and GALV GPs with that of MLV-A GP, which efficiently pseudotypes lentiviral vectors and, therefore, contains all the elements required for optimal incorporation on lentiviral particles, resulted in strongly increased incorporation of either glycoprotein on lentiviral cores ***(25,30,32)***. Such modifications preserve the host-range of the initial glycoproteins, as assessed on receptor-interference assays, and confer 10- to 100-fold increased titers to the SIV vectors. They may be useful for other glycoproteins that fail to be incorporated on lentiviral core particles.

Lentiviral vectors pseudotyped with the glycoproteins derived from several membrane-enveloped viruses present different advantages and potential properties in terms of both in vivo and ex vivo gene transfer applications (*see* **Note 2**). Finally, since the tropism of influenza virus hemagglutinin, MLV and GALV envelope glycoproteins can be changed ***(44–46)*** and because they can be used to pseudotype SIV vectors ***(3,31)***, it is possible to exploit the wide range of or develop novel receptor-targeted glycoproteins that have been engineered using these different glycoproteins in order to modify the host-range of lentivirus vectors ***(47,48)***.

1.5. Assembly of Viral Particles

Lentiviral vector are usually prepared from transient expression of the vector components in highly transfectable cells (e.g., 293T cells), although alternative and novel methods to generate SIV vectors rely on the use of inducible packaging cell line ***(49)***. The expression of both the transfer vector and the packaging vector(s) into cotransfected cells **(Fig. 1D)** allows the release in the culture supernatant of virus particles that have packaged the genome of the transfer vector and which are replication-defective. They can usually be

produced in large quantities and concentrated and purified before being used to transduce the target cells **(Fig. 1E)**. The recombinant virus retains the ability to enter into the cell via a specific receptor that is recognized by the viral glycoprotein and to integrate permanently its genetic material into the host genome, thus ensuring an efficient and long-term gene delivery. Since no replication-competent virus is used to complement the defective vector, retroviral-mediated gene transfer is a single-round operation. This results in the insertion of one to a few copies of the transgene to the host cell genome **(Fig. 1E)**.

2. Materials

2.1. Cells

1. 293 cells (American Type Culture Collection [ATCC] CRL-1573).
2. TE671 cells (human rhabdomyosarcoma, ATCC CRL-8805).
3. sMAGI macaque cells ***(50)***, permissive to SIVmac251 replication and harboring a stably integrated β-galactosidase (lacZ) transcription unit driven by the SIV/HIV tat-inducible HIV-1 LTR.

2.2. Cell Culture

1. DMEM (Dulbecco's modified Eagle medium) with 0.11 g/L sodium pyridoxine and pyridoxine (Invitrogen, France). DMEM is supplemented with 10% heat-inactivated (56°C; 1 h) South American fetal calf serum (FCS, Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), fungizone (Amphotericin B 0.250 μg/mL, Invitrogen). The final medium is stored at 4°C.
2. Phosphate-buffered saline (PBS) without calcium and magnesium, without sodium bicarbonate (Invitrogen).
3. Trypsin-ethylenediaminetetraacetic acid (EDTA) 1X in Hank's balanced salt solution without calcium and magnesium, with EDTA (Invitrogen).
4. Versene; 1:5000 (Invitrogen).
5. Bovine serum albumin (BSA, fraction V, Euromedex, France) is used at 1% in PBS as freezing medium and stored at +4°C.
6. Hygromycin B in PBS (50 mg/mL, Invitrogen) is stored at +4°C.
7. G418 (Geniticin, Invitrogen). Stock concentration is 100 mg/mL in H_2O, filtered through a 0.22-μm filter and stored at –20°C. sMAGI cells are grown in DMEM/10% FCS/hygromycin B (50 μg/mL)/G418 (200 μg/mL).

2.3. Virus Production-DNA Transfection

1. Plasmid DNA is purified using QIAfilter plasmid isolation kits (Qiagen, France).
2. 2X HEPES-buffered saline (HBS) and 2*M* $CaCl_2$: Calphos Mammalian Transfection Kit (Clontech, BD Biosciences, Le Pont-de Clair, France).

3. Polyethylene glycol (PEG): a 50% PEG 6000 (Fluka, L'Isle d'Abeau, France) solution is prepared in H_2O, autoclaved, and stored at 4°C.

2.4. Transduction and Analysis

1. Polybrene (Hexadimetrine bromide, Sigma): stock concentration of 8 mg/mL in H_2O, filtered through a 0.22-μm filter, aliquoted, and stored at –20°C. Polybrene is used at a final concentration of 6 μg/mL medium during infection.
2. Paraformaldehyde (Sigma): stock solution of 4% in PBS, stored at room temperature.
3. Glutaraldehyde (Sigma): stock solution of 0.5% in PBS, stored at 4°C.
4. X-GAL Buffer: is the addition of 0.23 m*M* sodium deoxycholic acid (monohydrate, Sigma); 2 m*M* $MgCl_2$ (Sigma); 5 m*M* potassium ferricyanide (Sigma); 5 m*M* potassium ferrocyanide (Sigma); 0.002% NP 40 (Roche Diagnostics, France) diluted in PBS. For sMAGI cells, pH has to be adjusted to 9.0 with NaOH.
5. X-GAL (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside, Euromedex): is diluted in dimethyl formamide (Sigma) to a final (60X) stock concentration of 40 mg/mL and stocked at –20°C. X-Gal is freshly diluted in the X-Gal buffer before cell coloration.

3. Methods

3.1. Production of Lentiviral Vectors

1. Day (–1): 2.4×10^6 293 cells are seeded the day before transfection in 10-cm plates in a final volume of 12 mL.
2. Day 0: Cotransfection of SIV packaging constructs (8.1 μg) with the transfer vector constructs (8.1 μg) and the envelope-expressing construct (2.5 μg) using GIBCO calcium-phosphate transfection system (*see* **Note 3**).
3. Day 1: 15 h after transfection, the medium is replaced with 8 mL of fresh medium per plate.
4. Day 2: 36 h after transfection, virus are harvested, centrifuged at low speed to remove cellular debris, filtered through 0.45-μm pore-sized membranes, and stored at –80°C (*see* **Note 4**).

3.2. Concentration and Storage of Lentiviral Vector Particles

1. Virions released in transfected cells supernatants are pelleted in 26-mL ultracentrifugation tubes (25 × 89 mm, polyallomer, Beckman, Coulter, Nyon, Switzerland). Virions are spun for 2 h at 110,000*g* at 4°C in a 70Ti Beckman rotor (*see* **Note 5**).
2. After centrifugation, supernatants are removed and the tube walls are rinsed with 5 mL PBS.
3. Tubes are then inverted on absorbant papers for 5 min, left to dry for another 5 min, and wiped.
4. Viral pellets are resuspended for 2 h in ice cold PBS supplemented with 1% BSA in 1/100 of the initial volume of the viral supernatant.

5. Virions are aliquoted in eppendorf tubes and stored at –80°C or in liquid nitrogen (*see* **Note 6**). Samples can be kept over 6 mo at –80°C without affecting the infectious titers.

3.3. Transduction Assays

1. Day (–1): TE671 or sMAGI target cells are seeded at a density of 4×10^5 per well in 6-well plates in a final volume of 2 mL (*see* **Note 7**).
2. Day 0: serial dilutions of vector preparations, diluted to 1 mL in DMEM-10% FCS, are added to the cells in the presence of 6 μg of polybrene/mL.
3. Cultures are incubated for 4 h or longer at 37°C. The vector-containing medium is replaced with 2 mL of fresh culture medium. Cells are incubated for 72 h at 37°C.
4. Day 3: the transduced cells are individualized in trypsine, fixed in 4% formaldehyde in PBS for 30 min and transferred in FACS tubes (Becton Dickinson, France). The percentage of green fluorescent protein (GFP)-positive cells is determined by Fluorescence-Activated Cell Sorter (FACS) Calibur analysis (Becton Dickinson).

3.4. Analysis of Transduction

1. Transduction efficiency is usually determined as the percentage of GFP-positive cells after transduction of 4×10^5 target cells with 1 mL of viral supernatant.
2. Infectious titers are provided as transducing units (TU)/mL and can be calculated by using the formula:

$$\text{Titer} = \%inf \times (4 \times 10^5/100) \times d$$

   in which d is the dilution factor of the viral supernatant and $\%inf$ is the percentage of GFP-positive cells as determined by FACS analysis using dilutions of the viral supernatant that transduce between 5 and 10% of GFP-positive cells.
3. MOIs (multiplicities of infection) is the ratio between infectious particles and target cells, which are required to optimally transduce target cells of interest and which are generally much less permissive to transduction than the cells used for titrations (*see* **Note 8**).
4. The choice of a particular promoter used in the internal transcription cassette of the vector should be based essentially on its expected properties in the target cells of interest (*see* **Note 9**). Most of the time, this internal promoter has little or no influence on the determination of the infectious titers of the vector stocks.

3.5. Detection of RCRs in Vector Preparations

To detect RCRs, two assays can be designed by using sMagi target cells, permissive to SIVmac251 replication and harboring a stably integrated lacZ transcription unit driven by the SIV/HIV tat-inducible HIV-1 LTR ***(50)***.

1. Day (–1): sMAGI target cells are seeded at a density of 4×10^5 per well in 6-well plates in a final volume of 2 mL (*see* **Note 7**).

2. Day 0: sMAGI cells are primarily infected with SIV vector particles (for conditions of infection, *see* **Subheadings 3.3.** and **3.4.**). Serial dilutions of vector preparations, diluted to 1 mL in DMEM-10% FCS, are added to the cells in the presence of 6 μg of polybrene/mL.
3. Cultures are incubated for 4 h, or longer, at 37°C. The vector-containing medium is replaced with 2 mL of normal culture medium. Cells are incubated for 72 h at 37°C.
4. Day 3: the transduced cells are individualized in trypsine. The first third of the cell suspension is used to determine the percentage of GFP-positive cells. Cells are fixed in 4% formaldehyde in PBS for 30 min and analysed by FACS. The second third is reseeded in 24-well plates in a final volume of 1 mL to check β-galactosidase expression. Cells are fixed when confluent with 1 mL PBS-0.5% glutaraldhehyde for 10 min, rinsed with 1 mL PBS, and colored with a X-Gal solution (pH 9.0) for 2 h at 32°C. The last third is reseeded in 6-well plates in a final volume of 2 mL and cells are maintained 10 to 15 d in culture to allow spreading of an eventual RCR. The capacity of recombinant retroviruses to undergo replication and amplification within this timeframe in these cells is controlled by using the reverse transcriptase (RT) activity (RT assay, Roche Diagnostics, Meylan, France) and the tat gene expression in sMAGI cells infected by wild-type SIVmac251.
5. Day 14: sMAGI target cells are seeded at a density of 4×10^5 per well in 6-well plates in a final volume of 2 mL.
6. Day 15: the supernatants of the infected primary target cells are then used to infect intact sMAGI cells as secondary target cells. 1 mL of the supernatants is incubated on the target cells for 4 h at 37°C. The medium is replaced with 2 mL of normal culture medium. Cells are incubated for 48 to 72 h postinfection at 37°C. This transduction is realized to assess the putative mobilization of the GFP-containing SIV vector and the potential presence of replication-competent retroviruses.
7. Detection of both GFP and β-galactosidase expression in the secondary target cells, detected 48–72 h postinfection, must remain negative in all experiments to demonstrate the absence of mobilization of the SIV vector and to show that the stocks of SIV vectors are devoid of both RCRs and tat-recombinant retroviruses. Additionally, RT activity must remain negative in the supernatants of both primary and secondary infected cells after several passages to establish the absence of RCRs in the SIV-vector preparations (*see* **Note 10**).
8. As a positive control for these evaluations, we used wild-type SIVmac251 virus.

4. Notes

1. Further studies have indicated that lentiviral vectors generated with the FPV hemagglutinin (HA) could not egress from producer cells after GP assembly. Indeed, when vector-producer cells expressing the FPV-HA were treated with neuraminidase, infectivity of HA-pseudotyped vectors was strongly increased by up to

100-fold. This enhancement correlated with a 50-fold increased production of viral particles in the supernatant of producer cells. This was induced by neuraminidase-mediated release of virions from the cell surface on which they were retained because of binding to sialic acid-containing cell surface molecules.

2. For example, in contrast to VSV-G pseudotypes, vectors generated with RD114 glycoproteins are particularly resistant to human sera ***(31)***, suggesting that the latter vectors could be particularly suitable for systemic gene delivery. Moreover, in contrast to the broad tropism of VSV-G-pseudotyped lentiviral vectors that may not be suitable for particular gene transfer applications in which cell type-specific gene delivery would be required; more selective tropisms can be achieved by taking advantage of the natural tropisms of glycoproteins derived from some membrane-enveloped viruses. For instance, the use of surface glycoproteins derived from viruses that cause lung infection and infect via the airway epithelia, like Ebola virus or Influenza virus, may prove useful for gene therapy of the human airway ***(27)***. Other recent reports have demonstrated that onco-retroviral and lentiviral vectors pseudotyped with the RD114 GP efficiently transduce human and canine $CD34^+$ cells ***(31,33–35,38)***. Likewise, a comparative study of pseudotyped SIV vectors used to transduce the rat retina in vivo have shown that vectors generated with VSV-G or FPV hemagglutinin were particularly useful ***(51)***.
3. Best results in terms of infectious titers, infectivity, and reproducibility are obtained when purifying the DNA by cesium chloride gradients, yet good results may also be obtained upon quick DNA purification by PEG or commercial DNA extraction kits.
4. The producer cells can be used for a further second harvest of lentiviral vectors keeping in mind that a lower yield of virus will be achived. 8 mL of fresh DMEM-10% FCS are added on cells for a second 24 h virus production.
5. Polyallomer tubes give better results than polycarbonate tubes. Beckman Ultra-clear tubes should not be used since virions remain bound to the tube walls.
6. Pellets may also be resuspended in PBS-1% glycerol in order to reduce protein concentration of the viral stock. This is particularly useful for in vivo gene delivery applications ***(51)***.
7. Transduction can also be performed by infection of target cells in suspension conditions. 4×10^5 target cells, detached by versene treatment, and suspended in 1 mL of DMEM-10% FCS containing a 2X concentrated solution of polybrene (12 μg/mL) are seeded in 6-well plates. 1 ml of virus dilution (in DMEM-10% FCS) is added. Infection is run overnight at 37°C.
8. Other investigators also normalize vector preparations by measuring capsid protein in viral supernatants, using an enzyme-linked immunoabsorbent assay (ELISA) assays. However, this method detects both infectious and noninfectious vector particles, as well as virionfree capsid proteins (infectious particles usually represent less than 1% of the physical particles), in contrast to normalization of infectious titers on the best permissive cells (TE671 or sMAGI cells). The

normalization of vector stocks has been a recurrent problem, particularly when comparing different vector backbones and/or pseudotyping glycoproteins. In fact, neither MOIs nor quantitation of capsid proteins can normalize vector stocks in an accurate manner. Indeed, normalization by infectivity can be biased by the receptor expression pattern of the indicator target cells, whereas normalization by capsid measurement may vary in a 10-fold range between independent vector preparations. In fact, we found less variation in infectious titers than in capsid amounts when we compared two vector stocks prepared independently.

9. For example, although the CMV promoter usually drives strong expression of the transgene in most cell types, including primary hematopoietic cells analyzed shortly afterward (3–6 d postinfection), it may lead to transgene expression shutoff when expressed long-term. Weaker yet stable promoters, such as that of EF1alpha or PGK, may therefore be preferred ***(6)***.
10. Other methods to detect replication-competent retroviruses include polymerase chain reaction (PCR) detection on transduced target cells of sequences pertaining to the packaging genomes ***(15)*** or functional rescue assays of transduced viral genes ***(43)***.

References

1. Nègre, D., Duisit, G., Mangeot, P.-E., Moullier, P., Darlix, J.-L., and Cosset, F.-L. (2002) Lentiviral vectors derived from simian immunodeficiency virus (SIV), in *Current Topics in Microbiology and Immunology*, Vol. 261 (Trono, D. ed.), Springer-Verlag, Heidelberg, Germany, pp. 53–74.
2. Naldini, L., Blömer, U., Gallay, P., et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.
3. Nègre, D., Mangeot, P., Duisit, G., et al. (2000) Characterization of novel safe lentiviral vectors derived from simian immunodeficiency virus (SIVmac251) that efficiently transduce mature human dendritic cells. *Gene Ther.* **7,** 1613–1623.
4. Clements, J. and Zink, M. (1996) Molecular biology and pathogenesis of animal lentivirus infections. *Clin. Microbiol. Rev.* **9,** 100–117.
5. Gardner, M., Endres, M., and Barry, P. (1993) The simian retroviruses SIV and SRV, in *The Retroviridae* (Levy, J., ed.), Vol. 3, Plenum Press, New York, NY, pp. 133–276.
6. Mangeot, P. E., Duperrier, K., Nègre, D., et al. (2002) High levels of transduction of human dendritic cells with optimized SIV vectors. *Mol. Ther.* **5,** 283–290.
7. Guan, Y., Whitney, J., Diallo, K. and Wainberg, M. (2000) Leader sequences downstream of the primer binding site are important for efficient replication of simian immunodeficiency virus. *J. Virol.* **74,** 8854–8860.
8. Das, A., Klaver, B., Klasens, B., Wamel, J. V., and Berkhout, B. (1997) A conserved hairpin motif in the R-U5 region of the human immunodeficiency virus type 1 RNA genome is essential for replication. *J. Virol.* **71,** 2346–2356.
9. Dorman, N. and Lever, A. (2000) Comparison of viral genomic RNA sorting mechanisms in human immunodeficiency virus type 1 (HIV-1), HIV-2, and moloney murine leukemia virus. *J. Virol.* **74,** 11,413–11,417.

10. Kaye, J. F. and Lever, A. M. (1998) Nonreciprocal packaging of human immunodeficiency virus type 1 and type 2 RNA: a possible role for the p2 domain of Gag in RNA encapsidation. *J. Virol.* **72,** 5877–5885.
11. Ilyinskii, P. and Desrosiers, R. (1998) Identification of a sequence element immediately upstream of the polypurine tract that is essential for replication of simian immunodeficiency virus. *EMBO J.* **17,** 3766–3774.
12. Giavedoni, L. and Yilma, T. (1996) Construction and characterization of replication-competent simian immunodeficiency virus vectors that express gamma interferon. *J. Virol.* **70,** 2247–2251.
13. Ruprecht, R. (1999) Live attenuated AIDS viruses as vaccines: promise or peril? *Immunol. Rev.* **170,** 135–149.
14. Patience, C., Takeuchi, Y., Cosset, F.-L., and Weiss, R. (1998) Packaging of endogenous retroviral sequences in retroviral vectors produced by murine and human packaging cells. *J. Virol.* **72,** 2671–2676.
15. Mangeot, P.-E., Nègre, D., Dubois, B., et al. (2000) Development of minimal lentiviral vectors derived from simian immunodeficiency virus (SIVmac251) and their use for the gene transfer in human dendritic cells. *J. Virol.* **74,** 8307–8315.
16. Schnell, T., Foley, P., Wirth, M., Munch, J., and Uberla, K. (2000) Development of a self-inactivating, minimal lentivirus vector based on simian immunodeficiency virus. *Hum. Gene Ther.* **11,** 439–447.
17. Wagner, R., Graf, M., Bieler, K., et al. (2000) Rev-independent expression of synthetic gag-pol genes of human immunodeficiency virus type 1 and simian immunodeficiency virus: implications for the safety of lentiviral vectors. *Hum. Gene Ther.* **11,** 2403–2413.
18. Nakajima, T., Nakamaru, K., Ido, E., Terao, K., Hayami, M., and Hasegawa, M. (2000) Development of novel simian immunodeficiency virus vectors carrying a dual gene expression system. *Hum. Gene Ther.* **11,** 1863–1874.
19. Stitz, J., Muhlebach, M. D., Blomer, U., et al. (2001) A novel lentivirus vector derived from apathogenic simian immunodeficiency virus. *Virology* **291,** 191–197.
20. Schneider, R., Campbell, M., Nasioulas, G., Felber, B. K., and Pavlakis, G. N. (1997) Inactivation of the human immunodeficiency virus type 1 inhibitory elements allows Rev-independent expression of Gag and Gag/protease and particle formation. *J. Virol.* **71,** 4892–4903.
21. Belshan, M., Park, G. S., Bilodeau, P., Stoltzfus, C. M., and Carpenter, S. (2000) Binding of equine infectious anemia virus rev to an exon splicing enhancer mediates alternative splicing and nuclear export of viral mRNAs. *Mol. Cell Biol.* **20,** 3550–3557.
22. Zufferey, R., Donello, J. E., Trono, D., and Hope, T. J. (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73,** 2886–2892.
23. Ott, D. E. (1997) Cellular proteins in HIV virions. *Rev. Med. Virol.* **7,** 167–180.
24. Swanstrom, R. and Wills, J. W. (1997) Synthesis, assembly, and processing of viral proteins, in *Retroviruses* (Coffin, J. M., Hughes, S. H., and Varmus, H. E., eds.), Cold Spring Harbor Laboratory Press, New York, pp. 263–334.

25. Christodoulopoulos, I. and Cannon, P. (2001) Sequences in the cytoplasmic tail of the gibbon ape leukemia virus envelope protein that prevent its incorporation into lentivirus vectors. *J. Virol.* **75,** 4129–4138.
26. Desmaris, N., Bosch, A., Salaun, C., et al. (2001) Production and neurotropism of lentivirus vectors pseudotyped with lyssavirus envelope glycoproteins. *Mol. Ther.* **4,** 149–156.
27. Kobinger, G. P., Weiner, D. J., Yu, Q. C., and Wilson, J. M. (2001) Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. *Nat. Biotechnol.* **19,** 225–230.
28. Lewis, B. C., Chinnasamy, N., Morgan, R. A., and Varmus, H. E. (2001) Development of an avian leukosis-sarcoma virus subgroup A pseudotyped lentiviral vector. *J. Virol.* **75,** 9339–9344.
29. Mochizuki, H., Schwartz, J. P., Tanaka, K., Brady, R. O., and Reiser, J. (1998) High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells. *J. Virol.* **72,** 8873–8883.
30. Salmon, P., Nègre, D., Trono, D., and Cosset, F.-L. (2000) A chimeric GALV-derived envelope glycoprotein harboring the cytoplasmic tail of MLV envelope efficiently pseudotypes HIV-1 vectors. *J. Gen. Med.* **2 (Suppl.),** 23.
31. Sandrin, V., Boson, B., Salmon, P., et al. (2002) Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34+ cells derived from human and non-human primates. *Blood* **100,** 823–832.
32. Stitz, J., Buchholz, C., Engelstadter, M., et al. (2000) Lentiviral vectors pseudotyped with envelope glycoproteins derived from gibbon ape leukemia virus and murine leukemia virus 10A1. *Virology* **273,** 16–20.
33. Gatlin, J., Melkus, M. W., Padgett, A., Kelly, P. F., and Garcia, J. V. (2001) Engraftment of NOD/SCID mice with human CD34+ cells transduced by concentrated oncoretroviral vector particles pseudotyped with the feline endogenous retrovirus (RD114) envelope protein. *J. Virol.* **75,** 9995–9999.
34. Goerner, M., Horn, P. A., Peterson, L., et al. (2001) Sustained multilineage gene persistence and expression in dogs transplanted with CD34(+) marrow cells transduced by RD114-pseudotype oncoretrovirus vectors. *Blood* **98,** 2065–2070.
35. Kelly, P., Vandergriff, J., Nathwani, A., Nienhuis, A., and Vanin, E. (2000) Highly efficient gene transfer into cord blood nonobese diabetic/severe combined immunodeficiency repopulating cells by oncoretroviral vector particles pseudotyped with the feline endogenous retrovirus (RD114) envelope protein. *Blood* **96,** 1206–1214.
36. Marandin, A., Dubart, A., Pflumio, F., et al. (1998) Retroviral-mediated gene transfer into human CD34+/38– primitive cells capable of reconstituting long-term cultures in vitro and in nonobese diabetic-severe combined immunodeficiency mice in vivo. *Hum. Gene Ther.* **9,** 1497–1511.
37. Movassagh, M., Desmyter, C., Baillou, C., et al. (1998) High-level gene transfer to cord blood progenitors using gibbon ape leukemia virus pseudotyped retroviral vectors and an improved clinically applicable protocol. *Hum. Gene Ther.* **9,** 225–234.

38. Porter, C. D., Collins, M. K. L., Tailor, C. S., et al. (1996) Comparison of efficiency of infection of human gene therapy target cells via four different retroviral receptors. *Hum. Gene Ther.* **7,** 913–919.
39. Peng, K. W., Pham, L., Ye, H., et al. (2001) Organ distribution of gene expression after intravenous infusion of targeted and untargeted lentiviral vectors. *Gene Ther.* **8,** 1456–1463.
40. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J. K. (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* **90,** 8033–8037.
41. Seganti, L., Superti, F., Girmenia, C., Melucci, L., and Orsi, N. (1986) Study of receptors for vesicular stomatitis virus in vertebrate and invertebrate cells. *Microbiologica* **9,** 259–267.
42. DePolo, N. J., Reed, J. D., Sheridan, P. L., et al. (2000) VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivated by human serum. *Mol. Ther.* **2,** 218–222.
43. Cosset, F.-L., Takeuchi, Y., Battini, J., Weiss, R., and Collins, M. (1995) High titer packaging cells producing recombinant retroviruses resistant to human serum. *J. Virol.* **69,** 7430–7436.
44. Hatziioannou, T., Delahaye, E., Martin, F., Russell, S. J., and Cosset, F.-L. (1999) Retroviral display of functional binding domains fused to the amino-terminus of influenza haemagglutinin. *Hum. Gene Ther.* **10,** 1533–1544.
45. Cosset, F.-L., Morling, F. J., Takeuchi, Y., et al. (1995) Retroviral retargeting by envelopes expressing an N-terminal binding domain. *J. Virol.* **69,** 6314–6322.
46. Fielding, A., Chapel-Fernandes, S., Chadwick, M., Bullough, F., Cosset, F.-L., and Russell, S. (2000) A hyperfusogenic Gibbon ape leukemia virus envelope glycoprotein: targeting of a cytotoxic gene by ligand display. *Hum. Gene Ther.* **11,** 817–826.
47. Lavillette, D., Russell, S. J., and Cosset, F.-L. (2001) Retargeting gene delivery by surface-engineered retroviral vector particles. *Curr. Opin. Biotechnol.* **12,** 461–466.
48. Russell, S. J. and Cosset, F.-L. (1999) Modifying the host range properties of retroviral vectors. *J. Gene Med.* **1,** 300–311.
49. Kuate, S., Wagner, R., and Uberla, K. (2002) Development and characterization of a minimal inducible packaging cell line for simian immunodeficiency virus-based lentiviral vectors. *J. Gene Med.* **4,** 347–355.
50. Chackerian, B., Haigwood, N. L., and Overbaugh, J. (1995) Characterization of a CD4-expressing macaque cell line that can detect virus after a single replication cycle and can be infected by diverse simian immunodeficiency virus isolates. *Virology* **213,** 386–394.
51. Duisit, G., Conrath, H., Saleun, S., et al. (2002) Five recombinant SIV pseudotypes lead to exclusive transduction of retinal pigmented epithelium in rat. *Mol. Ther.* **6,** 446–454.

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FIV Vectors

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1. Introduction

Lentiviral vectors combine two useful vector properties: permanent integration and transduction of nondividing cells. Replication-defective lentiviral vectors were originally derived entirely from the human lentivirus human immunodeficiency virus type 1 (HIV-1) ***(1,2)***. Application of pseudotyping with broadly tropic, physically stable envelope glycoproteins ***(3)*** permitted efficient transduction of nonlymphocytes by HIV-1 vectors in vitro and in vivo ***(4,5)***. The first nonprimate lentivirus-based vector was derived from feline immunodeficiency virus (FIV) ***(6)***. Subsequently, substantial improvements have been made in the design and capabilities of FIV vectors, and recently identified FIV elements (central polypurine tract, central termination sequence, and packaging signal) have been incorporated ***(7,8)***. This chapter will briefly present an overview of FIV vectors, describe recent improvements, and then explain practical methods for production and use.

1.1. Important Lentiviral Properties

Retroviral reverse transcription yields a linear double-stranded DNA intermediate that is integrated into the target cell genome in a reaction catalyzed by the viral integrase. Therefore, retroviral vectors generate permanent transgenes, a process that makes the former appealing for therapy of chronic diseases. This capability was first demonstrated for vectors derived from simple oncoretroviruses, e.g., murine leukemia viruses. Oncoretroviral vectors have now been incrementally optimized to the point that clinical utility has been demonstrated for diseases that are recognized to be the targets most accessible

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to gene therapy *(9,10)*. For example, children with common gamma chain deficiency, in which gene-altered cells have a marked survival advantage, have sustained clinical improvement *(10)*. However, it became apparent early on that onco-retroviral vectors achieve integration only in target cells that are proliferating at the time of transduction *(11,12)*. This limitation, which precludes targeting many clinically relevant cell types, is a consequence of the fact that the reverse-transcribed linear DNAs (preintegration complexes) of these simple retroviral vectors cannot traverse the intact nuclear envelopes of interphase cells. Completion of the replication cycle instead depends on breakdown of the envelope during mitosis *(11,12)*. In contrast, the lentiviral vector preintegration complex is imported through the nuclear pore, permitting integration in nondividing cells *(4,6,13–15)*. The capacity to infect nondividing cells is fundamental to the universal lentiviral strategy of propagating through terminally differentiated macrophages and has been the principal motivation for engineering viral vectors from these highly pathogenic viruses. The mechanism of nuclear import remains controversial, but it has been attributed to multiple determinants in virion proteins *(16)* and to a plus strand discontinuity, the central DNA flap, which results from lentiviral initiation of plus strand synthesis at two locations *(7,17)*.

1.2. FIV and FIV Vectors

Three groups of lentiviruses infect primates, ungulates, and felines respectively. Feline immunodeficiency virus infects 10–20% of domestic cat populations worldwide, as well as many free-roaming, nondomestic *Felidae*, but is pathogenic only for the domestic cat. **Fig. 1 (Top)** illustrates the genomic structure of FIV 34TF10 *(18)*, the clone used as the substrate for replication-defective FIV vector work to date. FIV has typical lentiviral morphology and genetic structure, although only three nonstructural genes (*vif*, *rev*, and *orf2*) are encoded in comparison to the six encoded by the primate lentiviruses *(19,20)*. Other peculiarities are a Rev response element (RRE) that overlaps the 3′ end of *env* rather than the SU-TM junction and a *pol*-encoded dUTPase, which may facilitate reverse transcription under conditions of low nucleotide tension *(21)*.

1.3. Primate vs Nonprimate

A detailed consideration of the relative safety profiles of HIV and FIV vectors is beyond the scope of this chapter. Indeed, while theoretical considerations can be marshaled on either side, empirical data are lacking. No lentiviral vector system will proceed to clinical use without documentation of effective mechanisms to prevent and to screen for the most recognized threat: replication-competent retrovirus generation. Nevertheless, there are some reasons to

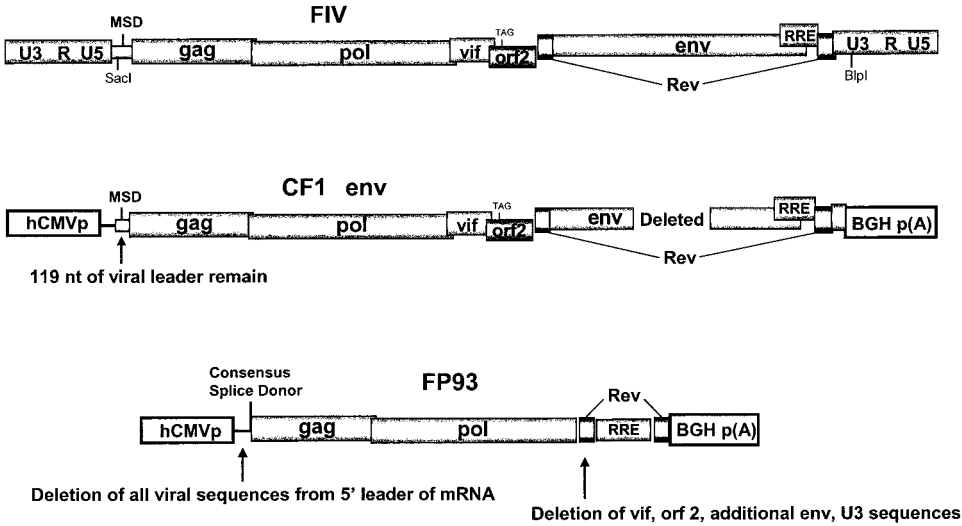

Fig. 1. Genomic structure of FIV and derivative packaging constructs. **Top:** Genome of FIV34TF10. LTR is long terminal repeat; U3, 3′ unique region of LTR; U5, 5′ unique region of LTR; R, repeat element of LTR; SD, major splice donor; Gag, group antigen (encodes structural components of virion core particle); Pol encodes a polyprotein that is cleaved by the viral protease into the five enzymatic activities: reverse transcriptase, integrase, RNAse H, protease, and dUTPase; Vif, virion infectivity factor; SU, surface envelope glycoprotein; TM, transmembrane portion of the envelope glycoprotein; RRE, rev response element. Orf2 is open reading frame 2. The Orf2 gene product may have LTR transactivating activity similar to HIV-1 Tat ***(49)***. However, ORF2 is not expressed by FIV 34TF10 because of the illustrated premature stop codon, and in any case, the vector system dispenses with the promoter activity of the FIV U3 element entirely by using a CMV promoter substitution and fusion at the TATA box (explained below). **Middle:** First generation packaging construct pCF1 *env*. **Bottom:** Second generation packaging construct pFP93. Note deletions of *vif*, *Orf2*, additional *env* sequences, and removal of all viral sequences upstream of *gag*. Deletions of *vif* and *orf2* are attenuating to FIV in vivo ***(50,51)***.

hypothesize that FIV vectors could eventually have higher acceptability for a broad range of applications. A practical consideration for vector workers and for potential gene therapy recipients is that FIV lacks any capacity to generate immunological cross-reactivity with HIV, for example in diagnostic HIV antibody enzyme-linked immunosorbent assays (ELISAs) ***(19,20,22)***. In addition, there is an extensive record of a lack of human infection or disease despite widespread human exposure to wild-type FIV via its principal and very efficient natural mode of interfeline transmission, biting (which frequently

transmits other pathogens to humans), and despite the ability of the wild-type virus to use a human chemokine receptor for entry ***(6,20,23)***. This epidemiological record of prevalent, direct, and efficient human inoculation without sequelae is unique among the lentiviruses. Another consideration is that FIV vectors can be tested in an animal model that is susceptible to disease causation by the parental virus.

FIV vectors efficiently complete the postentry stages of the infection cycle in nondividing human cells despite a severe block to transcription from the FIV long terminal repeat (LTR) and to other life cycle mechanisms that prevent productive replication ***(6,23–25)***. The replication-defective FIV vector ***(6)*** derived from the infectious clone FIV34TF10 ***(18)*** was the first demonstration of the feasibility of using a nonprimate lentivirus as a substrate for vectors. The negligible FIV expression observed in human cells ***(23)*** was an initial obstacle to transfer vector production. This was overcome by substitution of the 5′ FIV U3 (but not the 3′ U3) with a heterologous promoter by a fusion at the TATA box just upstream of the R repeat ***(23)***. The change enabled expression of high levels of FIV proteins and FIV vectors in human cells ***(6,23)***, revealing that previously suggested blocks to other productive phase functions, e.g., Rev ***(26)***, do not exist, at least in the relevant human cell lines. Use of a human cell line for clinical vector production (such as 293 cells, which have been approved for adenoviral vector production by the Food and Drug Administration, FDA) is important since feline vector producer cells would be unacceptable for human clinical application because of the risk of known and unknown adventitious agents. For example, feline cells harbor multiple copies of an inducible, xenotropic, replication-competent type C endogenous retrovirus (RD114) that can replicate in human cells, resists inactivation by human serum complement, and is related at the nucleotide sequence level to a primate retrovirus (baboon endogenous virus) ***(27–30)***.

The transcriptional silence of the FIV U3 in most human cell types provides a first level self-inactivating or SIN feature, as it is copied to both LTRs in the human target cell. A standard U3 deletion can also be made to produce a conventional SIN vector (unpublished data). In the initial studies, FIV vectors incorporating the hybrid 5′ promoter were shown to transduce dividing, growth-arrested, and postmitotic human targets ***(6)***. These results have been reproduced by others ***(31,32)***, and envelope glycoprotein-pseudotyped lentiviral vectors have now been derived from all three subgroups of primate lentiviruses, HIV-1, HIV type 2 (HIV-2), and simian immunodeficiency virus (SIV) ***(4,5,13–15,33–37)***, and from the ungulate group of lentiviruses ***(38–40)***. HIV-1 vector systems have so far received more extensive validation and molecular engineering for vector optimization ***(14)***, but there is growing interest in exploring the potential of nonprimate lentiviral systems. It should

be emphasized that no direct, methodologically rigorous comparisons of transducing efficiencies per particle in vivo are available for different lentiviral vector systems, or for that matter in human tissues, although equivalent efficacy per transducing units of FIV and HIV vectors has been demonstrated in one human organ ***(24)***.

1.4. FIV Vector System Design

Although valuable for establishing proof-of-principle, numerous aspects of the first generation FIV vector system were not optimal from the standpoints of safety and effectiveness, and we have now extensively re-engineered it. Designs have benefited from new knowledge about FIV replication as well as incremental optimization of the transfer vectors and packaging constructs ***(7,8)***. We use the term "second-generation" with the recognition that "generation" designations have been applied arbitrarily in the past to incremental modifications of HIV and FIV vectors. Although it has some rationale within a given system, this terminology does not convey relative levels of engineering between systems.

The basic design of our current system arranges for virion proteins to be expressed and supplied in *trans* to minimal FIV transfer vectors; the latter encode the recombinant genomes that are encapsidated. A nonlentiviral envelope glycoprotein is used to pseudotype the vector particles. Vesicular stomatitis virus glycoprotein G (VSV-G) has been the most commonly used envelope for this purpose ***(3)***, but a wide variety of others can be used depending on the indication. Because retroviral RNA genomes are packaged dimerically into particles and undergo homologous recombination at high rates during reverse transcription, a central goal of retroviral vector system design is to separate viral protein-coding elements from *cis*-acting elements needed for expression, encapsidation, dimerization, reverse transcription, and integration. In this way, the risk of replication-competent retrovirus (RCR) formation can be minimized. Since some *cis*-elements (packaging signals, the RRE, central DNA flap, the polypurine tract, and the proximal portion of U3) overlap sequences that encode viral proteins, it is not trivial to eradicate all coding sequences from transfer vectors.

1.5. Improvements in FIV Packaging Constructs

The initial packaging construct, pCF1Δ *env* ***(6)***, was made by simply blunting a SacI-BlpI fragment containing most of the viral genome into the polylinker of an hCMV (human cytomegalovirus) immediate early gene promoter expression plasmid and then deleting a 0.9 kb fragment of the *env* gene (**Fig. 1**, **Top** and **Middle**). The mRNA of the resulting construct has since been shown to be 97% defective for packaging compared to wild-type; segments of leader upstream of the SacI site are needed for encapsidation ***(8)***. Nevertheless, significant

undesirable overlap with *cis*-acting transfer vector sequences is present in pCF1Δ *env*. The lefthand LTR (U3, R, and U5 elements) and an additional 154 nt of the 5′ FIV leader upstream of SacI were deleted, but 119 nt of leader remain upstream of *gag*, including the interval between the major splice donor and *gag*. At the right hand junction, viral sequences terminate 37 nt downstream of the second exon of Rev and most of U3 (all of R and U5 are missing). The dispensable *vif* gene is also intact. To improve the construct, we have carried out a series of modifications to delete *cis*-acting sequences and unneeded viral coding sequences while preserving Gag/Pol expression. In contrast to pCF1Δ *env*, the resulting construct, pFP93, lacks all viral leader sequences, as well as *vif*, and contains less residual *env* sequence (**Fig. 1**, **Bottom**). At the 3′ end, viral sequences terminate with the stop codon of Rev. A summary of packaging construct modifications follows.

1.5.1. Deletion of Leader Sequence and Nonstructural Genes; High-Level Protein Production

We have deleted all of FIV sequences upstream of the *gag* gene, replacing them with only a 9 nt canonical splice donor sequence. We have also deleted *vif*, additional *env*, and U3 sequences. Splice donors and acceptor sites were selectively inserted between *pol* and *rev* to permit splicing. This minimized packaging construct (pFP93) produces two- to threefold higher levels of supernatant reverse transcriptase than pCF1Δ *env*.

1.5.2. Packaging Signal Exclusion

We have systematically mapped encapsidation (packaging) determinants in FIV genomic mRNA using RNAse protection ***(8)***. The data demonstrated that the packaging constructs lack necessary encapsidation determinants.

1.5.3. Development of Class I Integrase Mutants for Control Vectors

To establish that transgene expression in vivo occurs from integrated vector DNA and to provide a control for pseudotransduction (*see* **Note 1**), we have constructed single amino acid mutants of FIV integrase (D64V) **(Fig. 2)**. The aspartic acid residue mutated to valine at position 64 is required for integrase catalytic (DNA cleaving and joining) activity. It is a universally conserved participant in the catalytic center of retroviral integrases ***(41)***. Studies in our laboratory have shown that the D64V FIV integrase mutation selectively blocks integrase enzymatic activity without interfering with mechanisms of the viral life cycle, that is, proper folding and cleavage of the Gag/Pol precursor into the various structural proteins and enzymes, assembly into particles, and reverse transcription are intact. This integrase mutant phenotype is designated “class I” ***(41)***. In contrast, simple deletions and other point mutations in retroviral

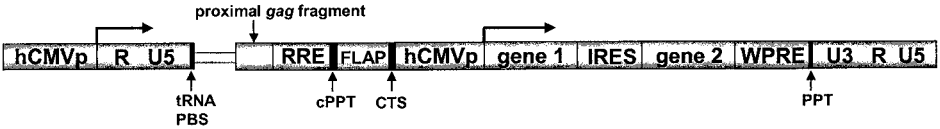

Fig. 2. Class I FIV integrase mutations. The three universally conserved amino acids (D64, D116, and E152) that are required for function of the integrase catalytic center are illustrated. The aspartic acid (D) at position 64 was mutated to valine (V) by site-directed mutagenesis. Subsequently, addition of a second mutation (D116A) has been shown to preserve class I properties.

integrase proteins generally result in class II phenotypes: pleiotropic effects on virion morphogenesis and other processes are observed. A packaging construct with a class I mutation permits generation of reverse transcriptase (RT)-normalized control FIV vectors that consist of viral particles identical to the active vector except for one amino acid in one internal, low molar constituent, the integrase. This allows precise functional study and direct examination of alternative endpoints to integration, e.g., one- and two-LTR circles. Subsequently, we have found that addition of a second mutation (D116A) to the D64V mutant preserves class I properties.

1.6. Improvements in FIV Transfer Vectors

As noted above, a fusion of the hCMV promoter to the R repeat permitted high-level expression of FIV proteins and production of infectious virus in human cells ***(6,23)***. This hybrid LTR is now the basis for transfer vectors. The initial transfer vector, CTRZlb, contained an hCMV-promoted lacZ gene cassette ***(6)***.

The most pertinent modifications to transfer vectors can be summarized:

1.6.1. Minimal Packaging Signal Inclusion

Complementary to the packaging construct changes, inclusion of much less of *gag* in the transfer vector (only 311 nt) has been enabled by mapping of the packaging signal, thereby reducing potential recombination with the packaging plasmid. In contrast, the original pCTRZLb transfer vector retained virtually all of *gag* (a frame shift was also inserted early in the reading frame). Moreover, we have found that inclusion of the smaller gag fragment enhances encapsidation compared to the original vector ***(8)***. Finer mapping of the amount of *gag* required is in progress.

1.6.2. Central DNA Flap

Vectors now include *cis*-acting elements that we have identified in FIV: the central polypurine tract (cPPT) and central termination sequence (CTS), which

are collectively termed the central DNA flap *(7)*. The cPPT and CTS are used in reverse transcription by FIV *(7)*, and the flap structure may be involved in enhancing nuclear import of the reverse-transcribed lentiviral DNA *(17)*. We have found that the central DNA flap is necessary for optimal infectivity and replication of FIV in vitro, and the addition of the flap to FIV transfer vectors increases titer in some cell lines (unpublished data); in vivo comparisons of flap (+) and (–) vectors are in progress.

1.6.3. Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE)

The WPRE, an RNA transport element that enhances transgene expression *(42,43)*, has been inserted upstream of the 3′ LTR.

1.6.4. Modular Construction

Modular construction, accessible cloning sites and cassettes oriented sense with respect to the transfer vector allow rapid adaptation to other transgenes.

1.6.5. Bi-cistronic Vector Utilizing an Internal Ribosomal Entry Site (IRES)

A bi-cistronic expression cassette with an IRES enables simultaneous expression of two transgenes. The IRES is a picornavirus element that mediates cap-independent translation, which obviates a need for multiple, potentially competing, internal promoters. Concentrated vector stock titers routinely exceed 1×10^9 on NIH3T3 fibroblasts for both IRES-linked reporter genes in vector pGiNWFlap **(Fig. 3)**.

1.7. Production of High-Titer FIV Vector Stocks by Transient Transfection of 293T Cells

At all stages of production and use, FIV vectors carrying marker genes and other genes lacking intrinsic hazard are handled in our institution at Biosafety Level 2 (BL2). Wild-type, infectious FIV is also classified as a BL2 agent *(44)*. For FIV vectors, the same general safety considerations that apply to onco-retroviral vectors pertain. In particular, transduction of known dominant oncogenes by any retroviral vector should only be carried out with stringent precautions to prevent exposure to personnel (generally BL3; *see* **ref.** *44* for additional information).

Like other laboratories, we use calcium phosphate cotransfection of plasmid DNA into 293T cells to produce lentiviral vectors. 293 cells were derived from human embryonic kidney by transfection of sheared adenoviral DNA, resulting in integrated copies of the adenovirus type 5 E1a and E1B genes *(45)*. 293T cells are a subsequent derivative that also express the SV40 T antigen. Previously these cells were considered to be derived from kidney epithelium. However, they resulted from outgrowth of extremely rare transformants from

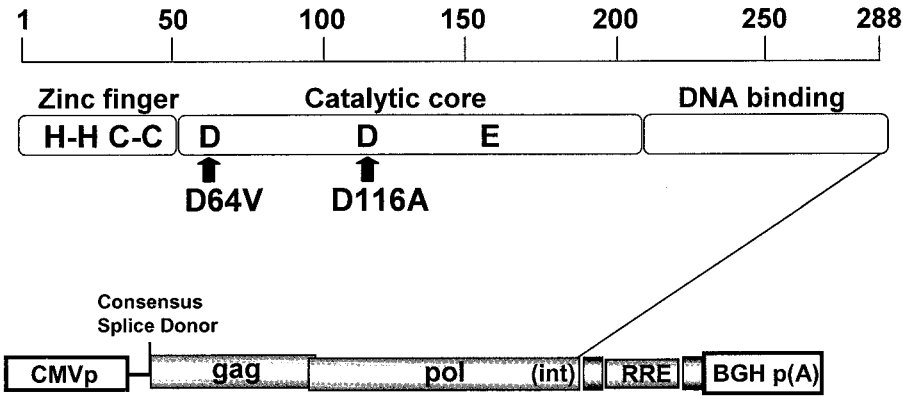

Fig. 3. Second generation bicistronic transfer vector. The central DNA flap *(7)* and WPRE *(42)* have been inserted, and *gag* has been reduced to 311 nt.

the source tissue, and recent evidence indicates that they are actually neuronal in origin and resemble neuronal progenitor/stem cells *(46,47)*. While the cells are highly transfectable, careful end optimization of the variable physicochemical process of DNA/hydroxyapatite nucleation has been found to be necessary to produce high quality lentiviral vector stocks. It should be emphasized that wide variations in vector titer have been found by many laboratories to result from relatively small variations in the transfection. Attention to final process optimization is essential. For example, varying the particular plasticware (flasks vs dishes or Cell Factories) can significantly influence effectiveness of a particular transfection regimen.

High efficiency 293T cell transfection permits rapid assessment of various transfer vectors and alternative envelope glycoproteins. Lentiviral vectors have been pseudotyped with a number of viral envelope glycoproteins. For many in vitro and in vivo gene-delivery experiments, pseudotyping FIV vectors with a rhabdoviral envelope such as mokola, rabies G, or VSV-G, has proven useful because rhabdoviral envelopes have broad tropism and are physically quite stable. VSV-G is the most widely used and has broad tropism (all vertebrate cells). The stability of particles pseudotyped with this protein permits concentration of vectors by ultracentrifugation. Other envelope glycoproteins, such as those of retroviruses (e.g., amphotropic envelope, gibbon ape leukemia virus) or arenaviruses, lymphocytic choriomeningitis virus (LCMV), may also be used depending on the cell target and are amenable to concentration in an ultracentrifuge with relatively little reduction in total yield.

The following protocol describes the scaled up production of VSV-G-pseudotyped FIV vectors and their concentration in a large volume, fixed angle

rotor for ultracentrifugation. Vector supernatants are made in multichamber Cell Factories (Nunc, Naperville, IL) with 1000 mL (CF10) or 200 mL (CF2) volumes. Use of Cell Factories accelerates vector production significantly. For example, with one CF10, the equivalent of 85 75-cm^2 flasks can be transfected in a single transfection. Centrifugation in six 220-mL buckets then allows 10- to 440-fold concentration of 1.32 liters in a single step, which can be followed by a second centrifugation step (a further 10- to 100-fold concentration) in a swinging bucket rotor if desired.

Alternatively, 75-cm^2 (T75) culture flasks can be used to produce 10-mL vector supernatant per flask. This is well suited to initial vector evaluation before scaled-up production. Both methods routinely yield 1 to 5 × 10^6 TU (EGFP-transducing units)/mL of GINWF, an FIV vector that carries enhanced fluorescent green protein (EGFP) and neomycin phosphotransferase (neoR) in the two IRES-linked positions shown in **Fig. 3**. The titers are determined by flow cytometry.

Many labs have found the large amount of high quality plasmid DNA required for lentiviral vector production to be quite burdensome (a total of 40–70 μg per 75 cm^2 of tissue culture surface is typically recommended in other protocols). The production method described here requires only 7 μg per 75 cm^2. We have found this less DNA-intensive protocol results in the same transfection efficiency and vector titer, while providing significant savings in time and expense for DNA preparation.

2. Materials

2.1. Cell Cultures

1. 293T cells.
2. Adherent fibro-epithelial cell lines for titration: HT1080, NIH3T3, CrFK cells.
3. Dulbecco's modified Eagle medium with 10% fetal calf serum (DMEM-10), penicillin G sodium (100 units/mL), streptomycin sulfate (100 μg/mL) and 2 m*M* L-glutamine.
4. Trypsin, tissue culture grade.
5. PBS (phosphate-buffered saline), tissue culture grade.
6. Distilled water, sterile.
7. 37°C humidified incubators, 5% CO_2.

2.1.1. Production in Cell Factories

1. Cell Factory with desired number of layers (Nunc Cell Factory, available as 1-layer CF1, 2-layer CF2, 10-layer CF10, and 40-layer CF40).
2. Cell Factory start-up kit (cat. no. 170769) with the following components: HDPE connectors (cat. no. 171838), white Tyvek cover caps (cat. no. 171897), blue sealing caps (cat. no. 167652), Gelman 4210 bacterial air vent filter.

3. 2 L Kimax aspirator bottle (Kimble Glass, Vineland, NJ, cat. no. 14607-2000) or similar bottle.
4. Funnels, sterile.
5. Cell strainers (BD Falcon, Franklin Lakes, NJ, cat no. 352350)

2.1.2. Production in T75 Flasks

1. Appropriate number of T75 tissue culture flasks with gas-permeable cap.

2.2. Replication-Defective, Tripartite FIV Vector System

1. pMD.G: plasmid encoding VSV-G: 84 μg for CF10, 16.8 μg (CF2), or 1 μg (T75).
2. Transfer vector. pGiNWF: minimal bi-cistronic FIV transfer vector plasmid coding for EGFP and neomycin phosphotransferase neoR, containing WPRE and FIV central DNA flap; pCT26: lacZ-encoding second generation FIV plasmid or other FIV transfer vector: 252 μg for CF10, 50.4 μg (CF2), or 3 μg (T75). This vector also has the central DNA flap inserted between the end of the *gag* segment and RRE.
3. pFP93: minimal FIV packaging plasmid coding for structural and enzymatic proteins derived from the Gag/Pol precursor (matrix, capsid, nucleocapsid, protease, reverse transcriptase, RNase H, integrase, dUTPase) and Rev (regulator of expression of virion proteins): 252 μg for CF10, 50.4 μg (CF2), or 3 μg (T75).

Ratio of pMD.G/pGiNWF/pFP93 is 1/3/3. DNA used for vector preparation for in vivo applications must be sterile and endotoxinfree to prevent toxic or inflammatory reactions in animals. There are several kits commercially available, EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany, cat. no. 2362). We generally use Qiagen, but cesium chloride purification is an alternative.

2.3. Transfections

1. 2.5 *M* $CaCl_2$.
2. 0.01 *M* Tris-HCl, pH 8.0.
3. 2X HBS (HEPES-buffered saline). Stock solution of dibasic Na_2HPO_4: 52.5*g* Na_2HPO_4, 5000 mL H_2O. 2X HBS: 80 g NaCl, 65 g N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (sodium salt), 100 mL Na_2HPO_4 stock solution. Bring volume to 5000 mL and adjust pH of 6.95, 7, and 7.05 with 1 *N* NaOH. Optimal pH needs to be determined (*see* **Note 5**): pH 6.95, 7.00, or 7.05.
4. Fresh culture media.

2.3.1. Cell Factories

1. Sterile plastic bottle (CF2, 250 mL; CF10, 500 mL).
2. Waste beaker with same volume as culture media in use.
3. Sterile Cell Factory loading bottle with silicon tube and connector.

2.3.2. T75 Flasks

1. Clear polystyrene 5-mL tubes (Falcon, cat. no. 352058).

2.4. Vector Harvest and Concentration

2.4.1. Cell Factories

1. 500-mL filter units, 0.22-μm pore size.
2. 250-mL polyallomer Oak Ridge ultracentrifuge bottles (Sorvall, Asheville, NC, cat. no. 54477) with fluorocarbon caps (Sorvall, cat. no. 54421) for A612 rotor (Sorvall, cat. no 11997).
3. Scale.
4. 36-mL disposable polyallomer ultracentrifuge tubes (Sorvall, cat. no. 03141) for SureSpin 630 rotor (Sorvall, cat. no. 79367).
5. 1.8-mL screw cap cryo vials, sterile.
6. 0.5- and 1.5-mL microcentrifuge tubes, sterile.
7. PBS, tissue culture grade/suitable for in vivo application.
8. 70% molecular grade ethanol in squeeze bottle.

2.4.2. T75 Flasks

1. Small filter unit (50 mL).

3. Methods

3.1. Preparation of 293T Cells

3.1.1. Cell Factory Seeding

See **Notes 2** and **3**.

3.1.1.1. For CF10 (*see* **Note 4**)

1. Day –6: Seed 4×10^6 293T cells into each of 4 T75 flasks.
2. Day –4: Trypsinize and seed 6.3×10^7 of the above 293T cells into a CF2.
3. Day –2: Trypsinize and seed a CF10 with 2.5×10^8 of the above 293T cells.
4. Day –1: Trypsinize the CF10 and reseed 2.5×10^8 of these 293T cells into the same CF10 (i.e., the original CF10 is reused). If desired and if full doubling occurred from day –2 to day –1, a second CF10 can be seeded.
5. Day 0: Transfection.

3.1.1.2. For CF2 (*see* **Note 4**)

1. Day –4: CF2: seed each of 4 T75 flasks with 3×10^6 293T cells.
2. Day –2: Trypsinize and seed a CF2 with 5×10^7 of the above 293T cells.
3. Day –1: Trypsinize and reseed 5×10^7 of the above 293T cells into a CF2s (the original is reused). If desired and if full doubling occurred over from day –2 to day –1, a second CF2 can be seeded.
4. Day 0: Transfection.

3.1.1.3. For T75 Flasks

1. Day –1: Seed 3×10^6 cells per flask. The cells will ideally have been split the day before seeding.
2. Day 0: Transfect.

3.2. Transfection and Vector Production (see Note 5)

3.2.1. For Cell Factories

1. Adjust DNA amount of the three plasmids pMD.G/pFP93/pGINWF to a ratio of 1/3/3. For CF10, use 84.5/253.5/253.5 μg in a 500-mL sterile plastic bottle, bring volume to 60.5 mL with 0.01 *M* Tris-HCl (pH 8.0), add 6.5 mL of 2.5 *M* $CaCl_2$, and mix by bubbling air into transfection mix with pipet. For CF2, use 16.9/50.7/50.7 μg in a 250-mL sterile plastic bottle, bring volume to 12.1 mL with 0.01 *M* Tris-HCl (pH 8.0), add 1.3 mL of 2.5 *M* $CaCl_2$, and bubble with pipet. Use of 0.01 *M* Tris-HCl is recommended over distilled water because ddH_2O may have an acid pH depending on the water supply.
2. Tilt bottle to gather contents in corner of bottle and add 67 mL (CF10) or 13.4 mL (CF2) of 2X HBS by rapid pipetting. Bubble air through the mix and and shake for 10 s. Vortexing is less desirable because contents are not mixed immediately. Set aside and let precipitate for exactly 3 min. The small DNA amounts used in this protocol result in fewer condensation nuclei during precipitation and faster growth of crystals than large DNA amounts.
3. While the precipitation continues, empty Cell Factory into waste beaker, wipe inlets again with 70% ethanol-soaked Kimwipe, and connect loading bottle with silicon tube and connector to Cell Factory. Fill loading bottle with 100 mL of fresh culture media per layer (total of 1000 mL in CF10 or 200 mL in CF2) without letting media run into Cell Factory.
4. At 3 min, stop precipitation by pouring transfection mix straight into media in loading bottle. Shake and bubble vigorously for even distribution.
5. Place Cell Factory on its side and fill by elevating loading bottle. Make sure chambers of Cell Factory contain equal media levels; lift Cell Factory at connector end while still placed on side to prevent media of upper chambers from leaking into lower chambers. Roll back into horizontal orientation. Put Cell Factory back into incubator in a precisely horizontal orientation.
6. Remove media 16 to 18 h later and replace with fresh media.

3.2.2. For T75 Flasks

1. 4 h before transfection replace old media with exactly 10 mL fresh media.
2. Adjust amount of DNA of pMD.G, pFP93 and pGINWF in a sterile plastic tube to 1, 3, and 3 μg. Bring volume with 0.01 *M* Tris-HCl (pH 8.0) to 800 μL and add 800 μL of 2.5 *M* $CaCl_2$ while vortexing at middle speed.
3. Let precipitate for exactly 3 min and pipet transfection mix all at once into media pooled at the end of the tilted T75. Mix at the end by turning the tilted flask back and forth around its longitudinal axis without dislodging cells.

4. Place in incubator and leave undisturbed until changing the medium, which should be done after 16–18 h (generally this interval is overnight).

3.3. Vector Harvest and Concentration

3.3.1. Cell Factories

1. On day 3, i.e., 48 h after replacement of transfection mix with fresh media, collect supernatant in large beaker, stir, and let sit for 3 min to allow detached cells to settle.
2. Filter through 0.22-μm filter into 500-mL filter units. One filter can be used for 300–400 mL vector supernatant before clogging. 0.45-μm filters do not result in higher titers but more debris will be pelleted during ultracentrifugation. Aliquot filtered vector supernatant into appropriate number of 1.8-mL cryovials. Pseudotyped FIV vectors are highly stable: halflives range from 24 (37°C) to 72 h (4°C).
3. Wash insides of 250-mL centrifuge buckets and lids with 70% ethanol. Aspirate dry with vacuum. Always fill buckets to top mark and use additional PBS if necessary. Air causes partial collapse of bottle during ultracentrifugation. Balance tubes on scale together with lids and close tightly.
4. Spin at $67{,}000g_{\mathrm{rmax}}$ for 6 h at 4°C. A brownish vector pellet will be visible at the outer bottom rim of the bucket; pellet is easier to detect after decanting. This particular rotor requires a longer spin time than the rotor used in the second round of concentration, because the maximum achievable g forces at the minimal radius are smaller ($18{,}000g_{\mathrm{rmin}}$).
5. Decant buckets, place on ice at 45° angle with pellet pointing upward, let sit on ice for 2 min, and suck up collected liquid at bottom. Rotate pellet to bottom and add 5 mL PBS if two rounds of concentration are planned. Use smaller volume if only one round of centrifugation is desired.
6. Start resuspension with 5-mL pipet by washing down the whole outer wall to which pellet is attached, along with the entire bottom and the outer rim. Direct the wash jet toward the pellet. This process should not take more than 5 min per bucket. Undispersed fragments are pipetted into 25-mL tubes for second round of concentration together with fully dispersed vector. This centrifugation and resuspension step will result in recoveries of 50–80%.
7. Aliquot some first round concentrated vector as desired in 150-μL fractions in tubes with narrow bottom, e.g., 500-μL microcentrifuge tube, to prevent drying during storage. Freeze at –80°C.
8. Wash insides of 36-mL centrifuge tubes, as well as buckets and lids of swinging bucket rotor with 70% ethanol. Suck dry with vacuum. Fill buckets to the maximum level with resuspended vector from first round. Add PBS if necessary. Balance tubes on scale together with lids and close tightly.
9. Spin at $67{,}000g_{\mathrm{rmax}}$, $31{,}000g_{\mathrm{rmin}}$ for 1 h 30 min at 4°C. A brown vector pellet will be visible at the bottom of the bucket.

10. Resuspend pellet in 500 μL PBS by washing the entire bottom with jets for 5 min. Set pipet to 250 μL and keep tip submerged to prevent bubbles and foam. Resuspension will result in a milky and slightly viscous liquid. Pipette resuspended vector into 1.5-mL microcentrifuge tube and spin for 3 min at 3000*g* to remove large unresuspended particles. The second ultracentrifugation and resuspension will result in only 30–50% recovery of vectors.
11. Aliquot vector in 50-μL fractions in tubes with narrow bottoms; freeze at –80°C.
12. Wash Cell Factories twice with dH_2O inside the hood. Seal and store at 4°C.

3.3.2. T75 Flasks

1. Collect supernatant 48 h after removal of the transfection mix and filter through 0.22-μm filter.
2. Aliquot into cryovials and freeze at –80°C.

3.4. Titration

1. Seed 2.5×10^5 CrFK or other adherent cell line (HT1080, HeLa, or NIH3T3) into each well of a 6-well plate.
2. Twenty-four hours later, thaw vector stocks and make 10-fold serial dilutions. These can be made in 24-well plates by serially passaging 200 μL into 1.8 mL of medium, taking care to mix by pipetting up and down (change the tip each time). For unconcentrated vector, 10^1- to 10^3-fold dilutions can be used. For singly concentrated vector, 10^2- to 10^5-fold dilutions are useful, and for doubly concentrated vector, 10^3- to 10^6-fold dilutions are used.
3. Remove media from 6-well plate and add each mixture to a well of the 6-well plate, either changing the tip each time or moving from most dilute to least dilute. Leave one well untransduced as a control. Note: Although 8 μg/mL of polybrene increases the transduction efficiency about 10-fold, it often cannot be used on sensitive cells in vitro and is not suitable for in vivo use. Because of this, titrating vectors with polybrene overestimates the effective titer during later applications and is not recommended.
4. After 6 h, replace supernatant with fresh media. Longer exposure is not toxic but does not result in more transducing units in the final readout.
5. EGFP: Fluorescence from EGFP-transduced CrFK cells can be first appreciated about 24 h after start of transduction. Trypsinize cells 48 h after start of transduction, wash with PBS, resuspend and fix in 1% formalin in PBS. Analyze percentage of transduced cells with Fluorescence-Activated Cell Sorter flow cytometry (FACS). Use of 1% formalin prevents quenching of fluorescence. Choose well with 10–30% EGFP-positive cells for most reliable computation of titer. Wells with more than 30% transduced cells likely contain a significant fraction of multiply transduced cells and lead to underestimation of titer when a linear calculation is used (*see* **Note 6**). Because FACS gives a percentage of

positive cells as a readout, the number of cells present at the time of transduction rather than at time point of seeding is properly used to calculate the correct titer. One round of cell division during 24 h is a reliable approximation for CrFK cells when cultured in standard DMEM-10. For a culture volume of 1 mL per well as tested here, calculate the titer as cells present at time point of transduction × percentage of FACS positive cells × dilution factor = transducing units per milliliter.

6. β-galactosidase: 48 h after transduction, fix cells with 1% glutaraldehyde for 3 min, wash with PBS, and incubate overnight at 37°C with 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) staining solution. Wash once with PBS and replace with 1% glutaraldehyde. Use a transparent 2-mm × 2-mm-square counting grid and determine number of squares per well. Count positive foci in 10 random squares at ×100 magnification and determine the number of positive colonies per well. Multiply count by dilution factor to obtain TU/mL. For a volume of 1 mL as tested here, the titer = total number of β-galactosidase positive colonies per well × dilution factor = transducing units per milliliter.
7. For transgenes that cannot be directly visualized by colorimetric assay or FACS, immunofluorescence to detect the protein may be used. Alternatively, direct linkage of the protein of interest to a marker gene via an IRES, as discussed in **Subheading 1.6.5.**, may be used. A less satisfactory method to provide a rough estimate is to determine RT activity of the vector and normalize it to that of a titered marker gene vector to estimate transducing units. Since there may be wide variations in TUs/RT unit (i.e., vector preparations vary in infectivity for a variety of reasons), this is only an indirect inference.

4. Notes

1. Pseudotransduction is false-positive transduction caused by carryover of preformed protein present in the vector preparation. This source of artifactual transduction needs to be considered in all lentiviral vector experiments. 293T cells cotransfected with VSV-G, a vesiculating protein, can release large amounts of preformed marker gene protein into the supernatant. Generally this results in a mottled, nonuniform appearance of the target cell, but this distinction is not always reliable, and in vivo in tissues it can be hard to distinguish from genuine *de novo* synthesis. Although enhanced green fluorescent protein (EGFP) can occasionally produce this problem, β-galactosidase (lacZ) is more likely to confound. Two controls for pseudotransduction can be used. First, the packaging plasmid can be omitted during vector production (transfer vector and VSV-G are transfected). This "mock" vector should be processed in parallel with the real vector. Run appropriate assays such as a western blot or X-Gal staining of lacZ-vector preparations to compare preparations and assure same amounts of preformed protein. The second, more elegant, control is the class I integrase mutant packaging plasmid discussed in **Subheading 1.5.3.**
2. For high transfection efficiency, 293T cells should be maintained at high passage frequency and should be rapidly and uniformly dividing prior to seeding for

transfection. This is the reason for the stepwise splitting and expansion at 24–48 h intervals indicated in the schedules given in **Subheading 3.1.** The schedule results in manageable, convenient cell numbers while saving time and expense for materials. One layer of a Cell Factory has a surface area of 632 cm^2 and should be seeded with 2.5×10^7 293T cells 24 h before transfection. Thus, 6 d (CF10) and 4 d (CF10 and CF2) before transfection 293T cells are seeded into proper culture vessel to allow quadrupling over 2 d.

3. For filling and emptying of Cell Factories, remove filter caps from both inlets. If needed, clean inlets with a 70% ethanol-soaked Kimwipe. Rapid loading during routine work can be accomplished with funnels inserted into an inlet. To passage cells wash once with PBS, add 2.5 ml trypsin per layer and digest for 5 min at 37°C. Add about 50 mL media per layer to flush cells out of Cell Factory and repeat as necessary. Cell clumps can easily be disrupted by forceful shaking in a 250-mL bottle for 20 s. Aspirate cell suspension from bottom and pipet with a 50-mL pipet through cell strainer to dissociate cells and remove clumps. Confirm complete dispersion of cell clumps under microscope. Should they persist, pipet against bottom of bottle while creating a tight seal at pipet tip.
4. Visualization of cells in bottom layers of CF10s is possible with the Nikon Eclipse TE300; cells in CF2s can be examined with most tissue culture microscopes. Cell Factories require a perfectly horizontal orientation to assure even cell density. A minimum of 100 mL media per layer is required. The factories can be reused several times and may be stored at 4°C for weeks when empty. For this purpose, remove media and clean by rinsing with sterile, distilled water (instead of PBS/trypsin), which will disrupt and wash out the cells.
5. Successful transfection depends on careful optimization of transfection conditions in the particular culture vessel. Scaling up of a well-working transfection protocol to larger volumes may fail because culture conditions are different. A key to successful transfection is an optimal pH of the 2X HBS. For this, prepare 5 L stocks of 2X HBS with pH 6.95, pH 7.00, and pH 7.05. One can use stuffer DNA in place of packaging and envelope plasmids, plus 5–10% transfer vector or other marker gene plasmid expressing EGFP in the transfection protocol described above. Forty-eight hours after removal of the transfection mix, which would be the time of harvest during a vector production, trypsinize cells and analyze by fluorescence-activated cell sorter (FACS). Choose 2X HBS that results in highest percent transfection. 90–100% transfection is required to obtain high titer vector preparations.
6. Since transduction is a stochastic process (the outcome of random collisions of viral particles with cells), some cells can be hit more than once, some multiple times, and some not all. The distribution of viral particles per susceptible cell in a population is best described by the Poisson distribution:

$$P(k) = (e^{-MOI})(MOI)^k/k!$$

where $P(k)$ is the fraction of cells infected by k virions ***(48)***. The actual multiplicity of infection (MOI) can be derived from the proportion of untransduced cells

in the culture, $P(0)$. If k is made 0 (i.e., if we ask how many cells in a population were stochastically not transduced), then the fraction of cells that receive 0 particles, $P(0)$, is given by:

$$P(0) = e^{-MOI}.$$

It follows that MOI = –ln $P(0)$.

The practical relevance of these equations is that the MOI required to transduce 5% of cells in a population is MOI = –ln(0.01) = 0.05 TUs per cell. However, as the MOI increases, the apparent linearity seen at low MOI disappears. The MOIs required to transduce 25, 63, 95, and 99% of cells are approx 0.29, 1.0, 3.0, and 4.6 TUs per cell respectively.

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References

1. Parolin, C. and Sodroski, J. (1995) A defective HIV-1 vector for gene transfer to human lymphocytes. *J. Mol. Med.* **73,** 279–288.
2. Poznansky, M., Lever, A., Bergeron, L., Haseltine, W., and Sodroski, J. (1991) Gene transfer into human lymphocytes by a defective human immunodeficiency virus type 1 vector. *J. Virol.* **65,** 532–536.
3. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J. K. (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* **90,** 8033–8037.
4. Naldini, L., Blomer, U., Gallay, P., et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.
5. Reiser, J., Harmison, G., Kluepfel-Stahl, S., Brady, R. O., Karlsson, S., and Schubert, M. (1996) Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proc. Natl. Acad. Sci. USA* **93,** 15,266–15,271.
6. Poeschla, E. M., Wong-Staal, F., and Looney, D. J. (1998) Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. *Nat. Med.* **4,** 354–357.
7. Whitwam, T., Peretz, M., and Poeschla, E. (2001) Identification of a central DNA flap in feline immunodeficiency virus. *J. Virol.* **75,** 9407–9414.
8. Kemler I., Barrraza R., and Poeschla, E. (2002) Mapping of the encapsidation determinants of feline immunodeficiency virus. *J. Virol.* **76,** 11,889–11,903.
9. Bonini, C., Ferrari, G., Verzeletti, S., et al. (1997) HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science* **276,** 1719–1724.

10. Cavazzana-Calvo, M., Hacein-Bey, S., de Saint, B. G., et al. (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288,** 669–672.
11. Miller, D. G., Adam, M. A., and Miller, A. D. (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell Biol.* **10,** 4239–4242.
12. Lewis, P. F. and Emerman, M. (1994) Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J. Virol.* **68,** 510–516.
13. Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. USA* **93,** 11,382–11,388.
14. Naldini, L. and Verma, I. M. (2000) Lentiviral vectors. *Adv. Virus Res.* **55,** 599–609.
15. Poeschla, E., Gilbert, J., Li, X., Huang, S., Ho, A., and Wong-Staal, F. (1998) Identification of a human immunodeficiency virus type 2 (HIV-2) encapsidation determinant and transduction of nondividing human cells by HIV-2-based lentivirus vectors. *J. Virol.* **72,** 6527–6536.
16. Fouchier, R. A. and Malim, M. H. (1999) Nuclear import of human immunodeficiency virus type-1 preintegration complexes. *Adv. Virus Res.* **52,** 275–299.
17. Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., and Charneau, P. (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101,** 173–185.
18. Talbott, R. L., Sparger, E. E., Lovelace, K. M., et al. (1989) Nucleotide sequence and genomic organization of feline immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **86,** 5743–5747.
19. Pedersen, N. C. (1993) The feline immunodeficiency virus, in *The Retroviridae*, Kluwer Academic Press, New York, NY, pp. 181–228.
20. Elder, J. H. and Phillips, T. R. (1993) Molecular properties of feline immunodeficiency virus (FIV). *Infect. Agents Dis.* **2,** 361–374.
21. Lerner, D. L., Wagaman, P. C., Phillips, T. R., et al. (1995) Increased mutation frequency of feline immunodeficiency virus lacking functional deoxyuridine-triphosphatase. *Proc. Natl. Acad. Sci. USA* **92,** 7480–7484.
22. Olmsted, R. A., Hirsch, V. M., Purcell, R. H., and Johnson, P. R. (1989) Nucleotide sequence analysis of feline immunodeficiency virus: genome organization and relationship to other lentiviruses. *Proc. Natl. Acad. Sci. USA* **86,** 8088–8092.
23. Poeschla, E. M. and Looney, D. J. (1998) CXCR4 is required by a nonprimate lentivirus: heterologous expression of feline immunodeficiency virus in human, rodent, and feline cells. *J. Virol.* **72,** 6858–6866.
24. Loewen, N., Fautsch, M. P., Peretz, M., et al. (2001) Genetic modification of human trabecular meshwork with lentiviral vectors. *Hum. Gene Ther.* **12,** 2109–2119.
25. Loewen, N., Bahler, C. K., Teo W., et al. (2002) Preservation of aqueous outflow facility after second-generation FIV vector-mediated expression of marker

genes in anterior segments of human eyes. *Invest. Ophthalmol. Vis. Sci.* **43(12)** 3686–3690.
26. Tomonaga, K., Miyazawa, T., Kawaguchi, Y., Kohmoto, M., Inoshima, Y., and Mikami, T. (1994) Comparison of the Rev transactivation of feline immunodeficiency virus in feline and non-feline cell lines. *J. Vet. Med. Sci.* **56,** 199–201.
27. McAllister, R. M., Nicolson, M., Gardner, M. B., et al. (1973) RD-114 virus compared with feline and murine type-C viruses released from RD cells. *Nat. New Biol.* **242,** 75–78.
28. McAllister, R. M., Nicolson, M., Gardner, M. B., et al. (1972) C-type virus released from cultured human rhabdomyosarcoma cells. *Nat. New Biol.* **235,** 3–6.
29. Takeuchi, Y., Cosset, F. L., Lachmann, P. J., Okada, H., Weiss, R. A., and Collins, M. K. (1994) Type C retrovirus inactivation by human complement is determined by both the viral genome and the producer cell. *J. Virol.* **68,** 8001–8007.
30. Neiman, P. E. (1973) Measurement of RD114 virus nucleotide sequences in feline cellular DNA. *Nat. New Biol.* **244,** 62–64.
31. Curran, M. A., Kaiser, S. M., Achacoso, P. L., and Nolan, G. P. (2000) Efficient transduction of nondividing cells by optimized feline immunodeficiency virus vectors. *Mol. Ther.* **1,** 31–38.
32. Johnston, J. C., Gasmi, M., Lim, L. E., et al. (1999) Minimum requirements for efficient transduction of dividing and nondividing cells by feline immunodeficiency virus vectors. *J. Virol.* **73,** 4991–5000.
33. Kim, S. S., Kothari, N., You, X. J., et al. (2001) Generation of replication-defective helper-free vectors based on simian immunodeficiency virus. *Virology* **282,** 154–167.
34. Chang, L. J., Urlacher, V., Iwakuma, T., Cui, Y., and Zucali, J. (1999) Efficacy and safety analyses of a recombinant human immunodeficiency virus type 1 derived vector system. *Gene Ther.* **6,** 715–728.
35. Mangeot, P. E., Negre, D., Dubois, B., et al. (2000) Development of minimal lentivirus vectors derived from simian immunodeficiency virus (SIVmac251) and their use for gene transfer into human dendritic cells. *J. Virol.* **74,** 8307–8315.
36. Schnell, T., Foley, P., Wirth, M., Munch, J., and Uberla, K. (2000) Development of a self-inactivating, minimal lentivirus vector based on simian immunodeficiency virus. *Hum. Gene Ther.* **11,** 439–447.
37. White, S. M., Renda, M., Nam, N. Y., et al. (1999) Lentivirus vectors using human and simian immunodeficiency virus elements. *J. Virol.* **73,** 2832–2840.
38. Olsen, J. C. (1998) Gene transfer vectors derived from equine infectious anemia virus. *Gene Ther.* **5,** 1481–1487.
39. Mitrophanous, K., Yoon, S., Rohll, J., et al. (1999) Stable gene transfer to the nervous system using a non-primate lentiviral vector. *Gene Ther.* **6,** 1808–1818.
40. Berkowitz, R., Ilves, H., Lin, W. Y., et al. (2001) Construction and molecular analysis of gene transfer systems derived from bovine immunodeficiency virus. *J. Virol.* **75,** 3371–3382.

41. Leavitt, A. D., Robles, G., Alesandro, N., and Varmus, H. E. (1996) Human immunodeficiency virus type 1 integrase mutants retain in vitro integrase activity yet fail to integrate viral DNA efficiently during infection. *J. Virol.* **70,** 721–728.
42. Donello, J. E., Loeb, J. E., and Hope, T. J. (1998) Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element. *J. Virol.* **72,** 5085–5092.
43. Zufferey, R., Donello, J. E., Trono, D., and Hope, T. J. (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73,** 2886–2892.
44. Biosafety in Microbiological and Biomedical Laboratories. 4th ed. 1999, Washington, D.C.: U.S. Department of Health and Human Services, Public Service and Centers for Disease Control and Prevention, National Institutes of Health.
45. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36,** 59–74.
46. Shaw, G., Morse, S., Ararat, M., and Graham, F. L. (2002) Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. *FASEB J.* **16,** 869–871.
47. Toth, K. and Wold, W. S. (2002) HEK? No! *Mol. Ther.* **5,** 654.
48. Flint, S., Enquist, L., Krug, R., Racaniello, V., and Skalkas, A. (2000) Virus cultivation, detection and genetics, in *Principles of Virology: Molecular Biology, Pathogenesis, and Control*, ASM Press, Washington, DC.
49. de Parseval, A. and Elder, J. H. (1999) Demonstration that orf2 encodes the feline immunodeficiency virus transactivating (Tat) protein and characterization of a unique gene product with partial rev activity. *J. Virol.* **73,** 608–617.
50. Pistello, M., Moscardini, M., Mazzetti, P., et al. (2002) Development of feline immunodeficiency virus ORF-A (tat) mutants: in vitro and in vivo characterization. *Virology* **298,** 84–95.
51. Inoshima, Y., Miyazawa, T., and Mikami, T. (1998) In vivo functions of the auxiliary genes and regulatory elements of feline immunodeficiency virus. *Vet. Microbiol.* **60,** 141–153.

21

Hybrid Lentivirus Vectors

Vicente Planelles

1. Introduction

Lentivirus vectors are promising tools for gene transfer ***(1,2)***. Retroviral vectors in general offer the unique advantage of stably integrating into the genome of the host cell, thus providing the basis for sustained gene expression. In contrast to the classical oncoretrovirus-derived vectors, lentivirus vectors are highly efficient at infection of nondividing cells. This unique ability is owing to the presence of nuclear localization signals on several virion associated proteins, which include matrix (MA), viral protein R (VPR), and integrase (IN) ***(3)*** and a *cis*-acting element termed the central polypurine tract (cPPT) ***(4,5)***.

Significant progress has been made in the development of lentiviral vectors. Currently, a three-element system is widely utilized with in vitro gene transfer experiments ***(1)***. This system uses three plasmids for virion production: (1) a transfer construct that harbors a therapeutic gene and/or a reporter, as well as also the necessary *cis*-acting sequences for RNA packaging, reverse transcription, and integration; (2) a packaging construct that provides viral proteins in *trans* (mainly Gag, Pol, Tat, Rev), with the exception of envelope; and (3) a third plasmid, which provides in *trans* a heterologous envelope glycoprotein.

Safety is a pressing issue that needs to be addressed before lentivirus vectors can be used in humans. In particular, the potential for recombination leading to replication-competent lentivirus (RCL), also referred to as "helper," is a concern with all retrovirus vectors. Generation of helper virus in preparations of replication-defective vectors has been documented in numerous instances involving oncoviruses ***(6–11)***. Helper virus has the potential for inducing pathogenesis as demonstrated by studies in which monkeys were infused

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with transduced bone marrow cells after ablation of endogenous marrow with gamma irradiation ***(7,10,11)***. In these studies, helper virus gave rise to lymphoma in monkeys.

In an effort to develop retroviral and lentiviral vectors that are less recombination prone than conventional systems, various laboratories have developed hybrid vectors. The retrovirus genome has several properties that make it inherently plastic in terms of genetic engineering possibilities. Retrovirus genomes are small (in the order of 10 kb), and can be contained within a single plasmid. The various genes of a retrovirus genome can be encoded by separate transcriptional units. In addition, elements of different retroviruses can be combined to form hybrid particles. Three types of hybrid particles have been utilized in the development of vectors for gene therapy. The most common hybrid type consists of a retroviral particle in which the transfer and packaging constructs derived from a given retrovirus are co-expressed with an envelope construct derived from a different virus. This type of hybrid has been extensively described and is more commonly referred to as an envelope pseudotype or simply a pseudotype. Notably, retroviruses can form envelope pseudotypes with members of their own family, but also with glycoproteins from nonretroviruses, such as the vesicular stomatitits virus (VSV) ***(12–14)***. Other glycoproteins can be used for pseudotyping lentiviral vectors, such as the human T-cell leukemia virus (HTLV) ***(15)***, murine leukemia virus (MLV) ***(16)***, and filovirus ***(17)*** envelope glycoproteins, as well as the rabies virus glycoprotein G ***(18)***.

A second type of hybrid vector incorporates *cis*-acting regulatory sequences from a heterologous retrovirus, which may have desirable properties. An example of this strategy was described by Kung et al. ***(19)***. In this study, inclusion of the long terminal repeat (LTR) from a murine leukemia virus, passaged in rhesus macaques, led to an increase in the expression levels of lentiviral vectors in human T lymphocytes.

This chapter will focus on a third type of hybrid, which involves the combination of genomic RNA from a given retrovirus and a packaging construct from a heterologous retrovirus. Retroviral particles that package a heterologous transfer RNA are referred to as RNA pseudotypes ***(20–22)***. RNA pseudotype vectors have the potential advantage for decreased risk of homologous recombination between the packaging and the transfer vectors because of sequence dissimilarity between the transfer and packaging constructs. However, since efficient encapsidation of RNA requires interactions between structural motifs in the Gag proteins and RNA structures in the retroviral genome, RNA pseudotypes are only possible when combining elements of closely related viruses.

Corbeau and co-workers ***(23)*** created an RNA pseudotype by using a human immunodeficiency virus type 1 (HIV-1) packaging cell line and an HIV type

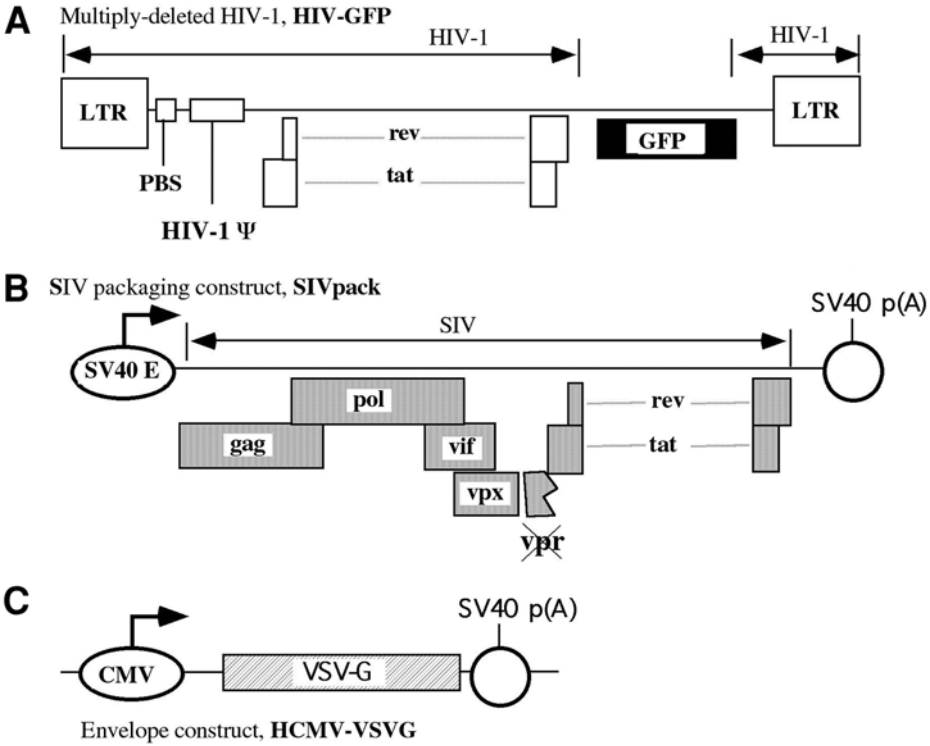

Fig. 1. Plasmids used in the production of an RNA pseudotype lentiviral vector. SIVpack is a packaging construct based on SIVmac1A11. SIVpack was constructed by subcloning a subgenomic fragment of SIVmac1A11 into an SV40-derived expression vector and subsequently deleting gp120 envelope sequences; SIVmac1A11 contains a frameshift mutation which inactivates *vpr*. HIV-GFP is a transfer vector based on HIV-1 and contains all the *cis*-acting elements needed for reverse transcription, integration, and expression. PBS, primer-binding site; LTR, long terminal repeat; Ψ, packaging sequence. These vectors were previously described ***(26)***.

2 (HIV-2) transfer vector. This HIV-1/HIV-2-based vector exhibited titers of 10^4 IU/mL and was capable of transducing nondividing cells such as primary human macrophages. Kaye et al. ***(24)*** showed that HIV-1-based packaging systems can efficiently package HIV-2 transfer constructs.

In a later system, described by White et al. ***(22)***, an HIV-1-derived transfer vector was encapsidated by simian immunodeficiency virus (SIV)mac virus core particles **(Fig. 1)**. These core particles were pseudotyped with the VSV glycoprotein G (VSV-G) **(Fig. 2A)**. The HIV-1-derived transfer vector, HIV-green fluorescent protein (GFP), expressed the GFP as a marker and, therefore, can be easily titrated by flow cytometry **(Fig. 2B)**. Encapsidation

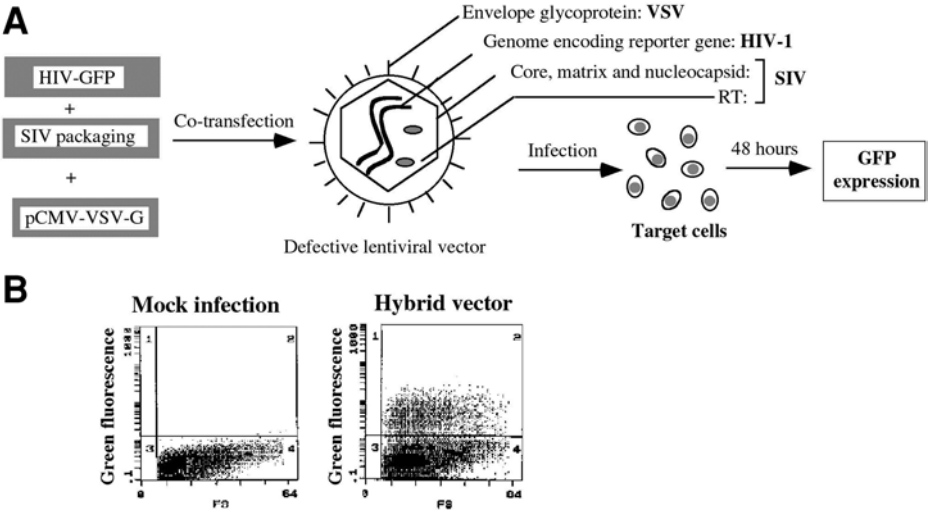

Fig. 2. Transient transfection of a three-plasmid system for production of a hybrid lentiviral vector. **(A)** A hybrid system using elements from SIVmac, HIV-1, and VSV glycoprotein G produces particles containing proteins and RNA from different viruses as shown. **(B)** Detection of transduced cells is accomplished by flow cytometric detection of fluorescent cells.

of a heterologous transfer vector by SlVmac-derived viral proteins is specific because it occurs when providing a transfer vector from a closely related virus, HIV-1 **(Table 1)**, but not a murine retrovirus, LNCX-GFP. When packaging DHIV-GFP with an HIV-1-derived packaging system, pCMVΔR8.2 ***(25)***, the titers are typically 20- to 50-fold higher than those obtained with the hybrid vector (data not shown). This RNA pseudotype vector system retained the ability to infect nondividing cells as evidenced when infecting radiation growth-arrested, cervical carcinoma HeLa cells ***(22)*** and human monocyte-derived macrophages **(Fig. 3)**. Recently, a similar system based on SIVagm was described, in which the SIVagm-derived transfer vector was efficienltly packaged by HIV-1 particles ***(26)***.

Because of its significant phylogenetic distance from primate lentiviruses, the feline immunodeficiency virus (FIV) is an attractive candidate from which gene therapy vectors can be developed. Interestingly, FIV can also cross-package its RNA genome into both SIV and HIV-1 capsid particles ***(27)***. Therefore, RNA pseudotypes combining feline and primate immunodeficiency virus elements are also feasible.

Table 1
Production of a Hybrid Lentiviral Vector

	Transfection constructs			
Name of virus	Transfer vector	Packaging vector	Envelope	IU / mL
HIV-GFP / SIVpack / G	HIV-GFP	SIVpack	HCMV-VSVG	2.3×10^6
HIV-GFP / Ψ(–)env(–)ampho / G	HIV-GFP	Ψ(–) env(–) ampho	HCMV-VSVG	< 100
HIV-GFP / SIVpack	HIV-GFP	SIVpack	None	< 100
HIV-GFP / G	HIV-GFP	None	HCMV-VSVG	< 100
LNCX-GFP / SIVpack / G	LNCX-GFP	SIVpack	HCMV-VSVG	< 100
LNCX-GFP / Ψ(–)env(–)ampho / G	LNCX-GFP	Ψ(–) env(–) ampho	HCMV-VSVG	1.3×10^5

HeLa cells (10^6) were infected with vectors at a dilution of 1:10 in tissue culture medium, and at 48 h postinfection; GFP-positive cells were quantitated by flow cytometry. Vector titers are in infectious units (IU) per milliliter. One infectious unit corresponds to one GFP-positive cell.

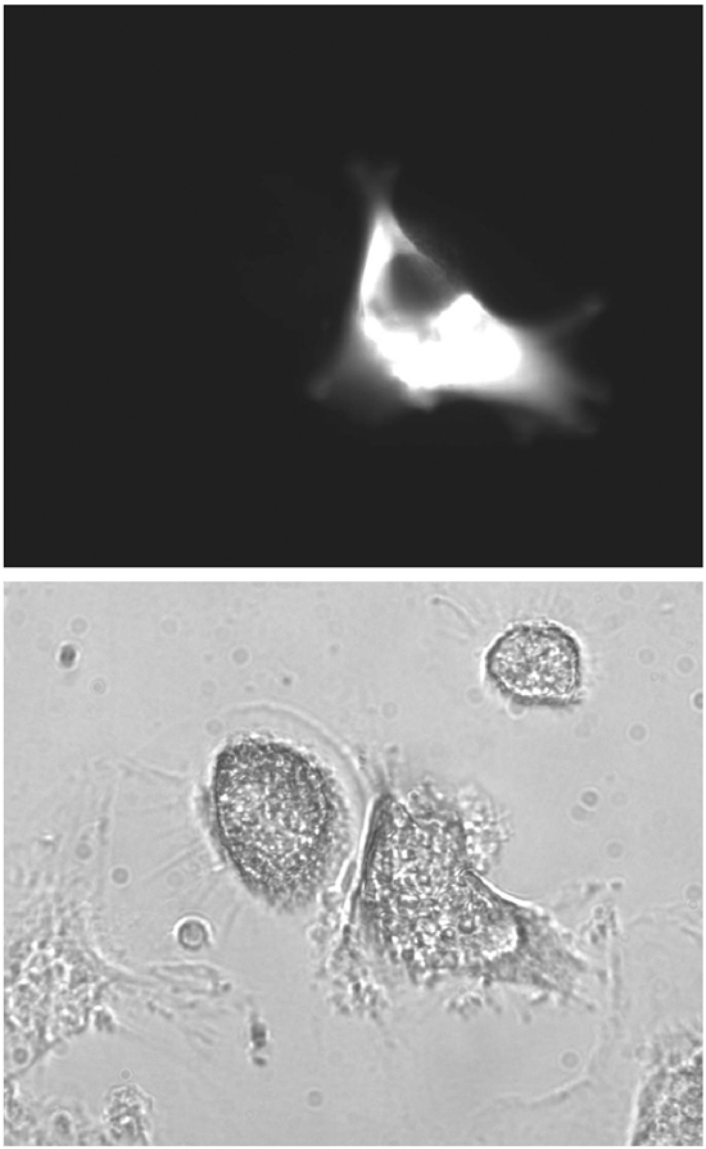

Fig. 3. Transduction of primary macrophages with a hybrid lentiviral vector. Human macrophages were isolated from peripheral blood and induced to differentiate by adherence to plastic, with addition of granulocyte-macrophage colony stimulating factor (GM-CSF), as described previously ***(26)***, and infected with a hybrid lentiviral vector. Cells were visualized by fluorescence microscopy (upper panel) and phase contrast (lower panel) at 48 h posttransduction.

2. Materials

2.1. Solutions and Reagents: Transfection by the Calcium Phosphate Precipitation Method

1. 2X HBS (HEPES-buffered saline) 100 mL: 1 g N-2-hydroxyethylpiperazine-N-ethanesulfonic acid (HEPES) (acid), 1.6 g NaCl, 0.72 mL Na_2HPO_4 (0.25 *M*), 1 mL KCl (1 *M*). Adjust pH to 7.1 with 5 *M* NaOH using 1 *M* NaOH for fine adjustments. Filter, alliquot solution, and store at 4°C (*see* **Note 1**).
2. 2 *M* $CaCl_2$: filter and store at 4°C.
3. 10 m*M* chloroquine: filter and store at –20°C (light sensitive).
4. Polybrene (Sigma, St. Louis, MO): 10 mg/mL in PBS, store at –20°C.
5. Fluorescence-Activated Cell Sorter (FACS) buffer: phosphate-buffered saline (PBS) with 2% fetal calf serum (FCS) and 0.02% sodium azide, 0.5 m*M* ethylenediaminetetraacetic acid (EDTA) (filter and store at 4°C).

2.2. Cell Culture Reagents and Cell Lines

1. Human embryonic kidney cell line, 293T (GeneHunter, Nashville, TN).
2. Human cervical cancer cell line, HeLa (American Type Culture Collection [ATCC], Manassas, VA) .
3. Dulbecco's modified Eagle medium (DMEM) (Bio-Whittaker, Walkersville, MD) supplemented with 10% FCS (Omega Scientific, Bedford, OH) plus penicillin and streptomycin.
4. 15-cm diameter tissue culture dishes (Corning, Corning, NY)
5. 10-cm diameter tissue culture dishes (Corning).
6. T-175 tissue culture flasks (Corning).
7. 10-mL disposable plastic pipets (Corning).

3. Methods

3.1. Calcium Phosphate-Mediated Transfection

1. Plate exponentially growing 293T cells in a 15-cm tissue culture dish. Pay close attention to the growth status of the cells (*see* **Notes 2** and **3**). When cells reach 60–70% confluent, perform transfection (*see* **step 2**). An adequate number of cells if plated the day before transfection is 5×10^6.
2. Feed the cells with 18 mL of fresh culture medium, brought up to room temperature.
3. Add 12.5 μg of transfer plasmid, 12.5 μg of packaging plasmid (SIVpack or pCMVΔR8.2), and 5 μg of envelope expression plasmid (the human cytomegalovirus promoter, HCMV-VSV-G) (for one dish) into a 50-mL conical tube and adjust the volume to 875 μL with sterile, double-distilled water (or other high purity water). Simply increase the volumes and amounts of DNA proportionally when transfecting multiple dishes.

4. Add 125 μL of 2 *M* $CaCl_2$ into the DNA solution, mix well, and put on ice for 5 min. Add 1000 μL of 2X HBS solution dropwise while applying vigorous agitation to the solution. Place on ice for 20 min.
5. Add the mixture to the cultured cells dropwise and gently rock the dish.
6. Add 100 μL of 10 m*M* chloroquine to the culture.
7. Incubate cell at 37°C with 5% CO_2 for 6–8 h. Longer incubation times may result in better transfection efficiencies (*see* **Note 4**). If possible, recommend a parallel transfection experiment with a GFP expression plasmid to check the quality of transfection solutions (*see* **Note 5**). Other transfection reagents can also be used (*see* **Note 6**).
8. Replace the medium with 20 mL of fresh medium. You should see a fine precipitation on the cells under microscope. Cells should be incubated for the next 36 h. Be aware that the cells become very fragile after transfection, and therefore, you should avoid moving the dish or flask.
9. On d 3 (36 h after removing transfection solution), harvest the medium and add back 20 mL of fresh culture medium to the cells. Centrifuge the harvested medium at 1000*g* in an RT7 centrifuge with an RTH-750 rotor (Sorvall, Newton, CT) for 10 min, collect the supernatant, and store at 4°C. This step of centrifugation removes cellular debris. Repeat collections daily up to d 7. A filtration step can be included (optional) before concentration (*see* **Note 7**).

3.2. Vector Concentration by Ultracentrifugation

1. Place the collected supernatant in ultracentrifuge tubes. The centrifuge chamber must be prechilled.
2. Centrifuge at 45,000*g*, 4°C for 2 h. Decant the supernatant leaving behind 200–300 μL (if you have extra collections to concentrate, you can reload the supernatant again to these tubes and perform 1–3 extra centrifugations). After the final round of concentration, discard the supernatant and resuspend the concentrated vector stock in 200 μL of fresh medium for each tube. Cap tubes with parafilm and place at 4°C overnight.
3. The next day, vortex briefly and pool all tubes of the same vector, aliquot, and freeze at –80°C (also *see* **Note 8**).

3.3. Vector Titration

Titration of viral vectors is best accomplished by monitoring expression of the reporter gene (if available) in the target cells. As target cells, HeLa cells are convenient because they can be grown quickly. However, vectors pseudotyped with VSV-G can infect most cell types, and therefore, the choices of target cells for titration are virtually unlimited.

1. Plate 1×10^6 HeLa cells in 10-cm tissue culture dishes. After cells are attached (this usually takes overnight incubation), incubate cells with 10 μg/mL polybrene in complete medium for 1 h.

2. Thaw the vector stock quickly in a 37°C water bath and prepare 3-mL inocula. Inocula are prepared by mixing complete medium plus 300, 30, or 3 μL of vector stock to a final volume of 3 mL, corresponding to 10-, 100-, and 1,000-fold dilutions, respectively. Remove medium form cells, add the inocula, and place dishes in the incubator. For a high-infection protocol, use the "spin infection" method as described in previous chapters of this book, as well as in literature ***(27,28)***.
3. On d 2, change the medium and culture for another 24 h.
4. On d 3, detach cells by incubation in 2 m*M* EDTA in 1X PBS. To examine the GFP expression, cells are gently centrifuged (1000*g*) and fixed in 0.2% paraformaldehyde (2% stock in 1X PBS). The titer can be calculated according to the following formula:

$$\text{Titer} = [F \times C_0 / V] \times D$$

F = frequency of GFP positive cells by flow cytometry; C_0 = total number of target cells at the time of infection; V = volume of inoculum; D = virus dilution factor. Alternatively, the titer can be measured by visual inspection under the fluorescence microscope.

4. Notes

1. For calcium phosphate-mediated transfection, it is critical to adjust the pH of the HBS solution precisely. Slight deviations from pH 7.1 will have a negative effect on the transfection efficiency.
2. For the production of lentiviral vectors, 293T cells are grown in DMEM with 10% FCS plus antibiotics. These cells should not be passaged for periods exceeding 2 mo. After 2 mo in culture, 293T cells should be discarded and a new, low-passage vial should be thawed for future transfections. Cells are typically competent for transfection 1 wk after being thawed. 293T cells can be passaged in medium containing either heat-inactivated or noninactivated FCS. However, we find that cells passaged in noninactivated serum produce higher lentivirus vector titers.
3. Transfection efficiency depends on the degree of confluence of the cells. In our hands, 60–70% confluence at the time of transfection produces the best results.
4. The DNA mixture should be incubated on the cells for at least 6 h. Alternatively, an overnight incubation can be performed, as long as it does not exceed 16 h. However, this incubation can, and should be, shortened if massive cell death is observed.
5. If possible, always perform a parallel transfection with a plasmid expressing GFP. Twenty-four hours following transfection, check the GFP expression levels. If the control transfection shows about 40% or higher efficiency, we consider the transfection acceptable.
6. Other transfection reagents, such as Lipofectamine 2,000 (Invitrogen, Carlsbad, CA) or TransFast (Promega, Madison, WI) can be used as alternatives to the

calcium phosphate method. These methods are typically less labor-intensive than the calcium phosphate method and comparable in efficiency. However, when performing large numbers of transfections, the calcium phosphate method provides a more economical option.

7. Alternatively, before the ultracentrifugation, the precleared supernatant can be filtered through a 0.45-μm pore size filter.
8. The vector stocks are very stable at –80°C. Avoid multiple rounds of freeze-thaw as the titer will drop significantly. Concentrated vector stocks can be kept at 4°C for up to 1 wk.

References

1. Klimatcheva, E., Rosenblatt, J. D., and Planelles, V. (1999) Lentiviral vectors and gene therapy. *Front. Biosci.* **4,** D481–D496.
2. VandenDriessche, T., Naldini, L., Collen, D., and Chuah, M. K. (2002) Oncoretroviral and lentiviral vector-mediated gene therapy. *Methods Enzymol.* **346,** 573–589.
3. Bukrinsky, M. I. and Haffar, O. K. (1997) HIV-1 nuclear import: in search of a leader. *Front. Biosci.* **2,** d578–d587.
4. Follenzi, A., Ailles, L. E., Bakovic, S., Geuna, M., and Naldini, L. (2000) Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat. Genet.* **25,** 217–222.
5. Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., and Charneau, P. (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101,** 173–185.
6. Chong, H. and Vile, R. G. (1996) Replication-competent retrovirus produced by a 'split-function' third generation amphotropic packaging cell line. *Gene Ther.* **3,** 624–629.
7. Donahue, R. E., Kessler, S. W., Bodine, D., et al. (1992) Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer. *J. Exp. Med.* **176,** 1125–1135.
8. Markowitz, D., Goff, S., and Bank, A. (1988) Construction and use of a safe and efficient amphotropic packaging cell line. *Virology* **167,** 400–406.
9. Otto, E., Jones-Trower, A., Vanin, E. F., et al. (1994) Characterization of a replication-competent retrovirus resulting from recombination of packaging and vector sequences. *Hum. Gene Ther.* **5,** 567–575.
10. Purcell, D. F., Broscius, C. M., Vanin, E. F., Buckler, C. E., Nienhuis, A. W., and Martin, M. A. (1996) An array of murine leukemia virus-related elements is transmitted and expressed in a primate recipient of retroviral gene transfer. *J. Virol.* **70,** 887–897.
11. Vanin, E. F., Kaloss, M., Broscius, C., and Nienhuis, A. W. (1994) Characterization of replication-competent retroviruses from nonhuman primates with virus-induced T-cell lymphomas and observations regarding the mechanism of oncogenesis. *J. Virol.* **68,** 4241–4250.

12. Akkina, R. K., Walton, R. M., Chen, M. L., Li, Q. X., Planelles, V., and Chen, I. S. (1996) High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *J. Virol.* **70,** 2581–2585.
13. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J. K. (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* **90,** 8033–8037.
14. Yee, J. K., Miyanohara, A., LaPorte, P., Bouic, K., Burns, J. C., and Friedmann, T. (1994) A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes. *Proc. Natl. Acad. Sci. USA* **91,** 9564–9568.
15. Landau, N. R., Page, K. A., and Littman, D. R. (1991) Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. *J. Virol.* **65,** 162–169.
16. Page, K. A., Landau, N. R., and Littman, D. R. (1990) Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. *J. Virol.* **64,** 5270–5276.
17. Kobinger, G. P., Weiner, D. J., Yu, Q. C., and Wilson, J. M. (2001) Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. *Nat. Biotechnol.* **19,** 225–230.
18. Reiser, J. (2000) Production and concentration of pseudotyped HIV-1-based gene transfer vectors. *Gene Ther.* **7,** 910–913.
19. Kung, S. K., An, D. S., and Chen, I. S. (2000) A murine leukemia virus (MuLV) long terminal repeat derived from rhesus macaques in the context of a lentivirus vector and MuLV gag sequence results in high-level gene expression in human T lymphocytes. *J. Virol.* **74,** 3668–3681.
20. Embretson, J. E. and Temin, H. M. (1987) Lack of competition results in efficient packaging of heterologous murine retroviral RNAs and reticuloendotheliosis virus encapsidation-minus RNAs by the reticuloendotheliosis virus helper cell line. *J. Virol.* **61,** 2675–2683.
21. Rizvi, T. A. and Panganiban, A. T. (1993) Simian immunodeficiency virus RNA is efficiently encapsidated by human immunodeficiency virus type 1 particles. *J. Virol.* **67,** 2681–2688.
22. White, S. M., Renda, M., Nam, N. Y., et al. (1999) Lentivirus vectors using human and simian immunodeficiency virus elements. *J. Virol.* **73,** 2832–2840.
23. Corbeau, P., Kraus, G., and Wong-Staal, F. (1998) Transduction of human macrophages using a stable HIV-1/HIV-2-derived gene delivery system. *Gene Ther.* **5,** 99–104.
24. Kaye, J. F. and Lever, A. M. (1998) Nonreciprocal packaging of human immunodeficiency virus type 1 and type 2 RNA: a possible role for the p2 domain of Gag in RNA encapsidation. *J. Virol.* **72,** 5877–5885.

25. Naldini, L., Blomer, U., Gallay, P., et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.
26. Stitz, J., Muhlebach, M. D., Blomer, U., et al. (2001) A novel lentivirus vector derived from apathogenic simian immunodeficiency virus. *Virology* **291,** 191–197.
27. Browning, M. T., Schmidt, R. D., Lew, K. A., and Rizvi, T. A. (2001) Primate and feline lentivirus vector RNA packaging and propagation by heterologous lentivirus virions. *J. Virol.* **75,** 5129–5140.
28. Kotani, H., Newton, P. B., III, Zhang, S., et al. (1994) Improved methods of retroviral vector transduction and production for gene therapy. *Hum. Gene Ther.* **5,** 19–28.

22

Cells of Respiratory Epithelium

Patrick L. Sinn and Paul B. McCray, Jr.

1. Introduction

The goal of correcting such genetic lung diseases as cystic fibrosis could be achieved by vector-mediated gene transfer to airway epithelia. The principle of using viral vectors to complement genetic defects is well documented in cell culture and in animal models. Moreover, strategies for the successful implementation of gene transfer-based therapies for patients are beginning to emerge ***(1)***. However, the airway epithelium has evolved many defensive barriers against microbial invasion ***(2,3)***. Indeed, these same barriers likely impede gene transfer efficiency with viral and nonviral vectors ***(3)***.

The research approaches outlined in this chapter focus on the use of nonprimate lentiviral vectors derived from feline immunodeficiency virus (FIV) ***(4,5)***. However, the protocols themselves are adaptable to a variety of viral vector systems. An optimal airway vector system would have the capacity to transduce readily pulmonary epithelial progenitor cells from the luminal surface and confer persistent expression with low immunogenicity. Although no truly ideal vector system exists, we have focused our studies on a nonprimate lentivirus-based vector for several reasons. As with all retroviral vectors, lentivirus-based vectors stably integrate into the host genome, an important consideration when the goal is to correct a chronic genetic disease. Additionally, in contrast to retroviral vectors such as Moloney murine leukemia virus (MLV), lentivirus-based systems can transduce mitotically quiescent airway cells ***(5–10)***. All viral vectors are subject to the challenge of overcoming the host's innate and adaptive immune responses; however, retroviral vectors appear to be less immunogenic than encapsidated vectors, and this characteristic potentially allows for readministration ***(11)***.

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Wild-type FIV is genetically and antigenically distinct from human immunodeficiency virus (HIV) and does not infect human cells or cause disease in humans ***(12)***, and therefore, FIV-based vectors may offer additional safety features over HIV-based systems ***(5)***. An FIV-based vector was first described by Poeschla et al. in which the 5′ viral long terminal repeat (LTR) U3 was replaced with the cytomegalovirus (CMV) promoter to allow transduction of human cells ***(4)***, and later, a second generation FIV-based vector was developed by Johnston and colleagues. The second generation FIV vector inactivated unnecessary elements (*vif*, *orf2*), further reducing the possibility of replication-competent virus production ***(5)***. To allow the recombinant FIV-based vector to transduce mammalian cells, the native envelope is replaced by pseudotyping with glycoproteins such as the vesicular stomatitis virus G glycoprotein (VSV-G) ***(13)***. A full discussion of the FIV vector preparation methods was reported in Chapter 20.

Although VSV-G is well documented to efficiently pseudotype lentiviral vectors, the choice of an envelope glycoprotein with the capacity to target appropriate airway cell types remains an important topic. To achieve the goal of persistent transgene expression to correct a lifelong genetic disease, a viral vector must target a population of cells with progenitor capacity. Therefore, the airway cells with progenitor capacity must be identified, and vectors must be optimized to target these cells. These cell types may include basal cells, intermediate cells, and some ciliated surface cells ***(14–17)***. The identification of envelopes with the capacity to target lentiviral vector entry via the apical surface in these cell types requires further research. However, many glycoproteins from enveloped viruses are under investigation as pseudotypes for retroviral vector targeting of the airway epithelium ***(18–23)***.

The respiratory epithelium has evolved a series of barriers to prevent viral infections, and therefore, functional examination of viral vectors in model systems that manifest these obstacles is required. Such barriers to gene transfer may include, but are not limited to (1) a mucus layer secreted by the epithelium designed to bind and clear foreign inhaled particles, (2) a ciliated surface, (3) airway surface liquid secreted by the cells, (4) an extracellular glycocalyx that may deter viral binding to cell surface receptors, and (5) an apical cell membrane lacking receptors for many viral vectors. Furthermore, potential barriers do not end with receptor binding. Viral entry may be impeded by the low basal rate of endocytosis at the apical surface, and a number of subsequent intracellular barriers to transduction may follow, including interference with reverse transcription, intracellular trafficking, translocation into the nucleus, and genomic integration ***(24,25)***. Although important data addressing some of these issues have been discovered using in vitro model systems, including immortalized cell lines and primary cultures, in vivo verification of in vitro

Table 1
To Prepare 10 mL of X-Gal Solution

Volume	Final concentration	Stock
8.3 mL		100 m*M* Tris-HCl, pH 8.0
0.7 mL	35 m*M*	0.5 *M* $K_3Fe(CN)_6$ (Protect from light)
0.7 mL	35 m*M*	0.5 *M* $K_4Fe(CN)_6 \cdot 3H_2O$ (Protect from light)
0.02 mL	2 m*M*	1 *M* $MgCl_2$
0.01 mL	0.01%	10% Sodium deoxycholate
0.02 mL	0.02%	10% NP40
0.25 mL	1 mg/mL	40 mg/mL X-Gal stock[a]

[a]X-Gal Stock: Dissolve 400 mg X-Gal (Fisher, cat. no. BP1615-1) in 10 mL dimethyl sulfoxide (DMSO). Protect from light. Store in 1-mL aliquots at –20°C.

observations is vital. The remaining sections of this chapter will focus on protocols designed to deliver viral vectors to the airways of animal models.

2. Materials

2.1. Mouse Procedures

2.1.1. Chemicals

1. Halothane (Halocarbon, River Edge, NJ) or ketamine (72 mg/mL)/xylazine (11.7 mg/mL)/acepromazine (1.7 mg/mL) (Abbott, Abbott Park, IL) for anesthesia.
2. Ethylene glycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid (EGTA) (Fisher Scientific, Atlanta, GA, cat. no. S311-500) for use in vector formulation.
3. Omnipaque (300 mg/mL; Novaplus, Irving, TX), a water soluble radio-opaque dye.
4. 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) solution (*see* **Table 1**).
5. Pentobarbital sodium solution, 300 mg/mL, for euthanasia.

2.1.2. Supplies

1. 22- or 24-gauge Teflon IV catheters for tracheal intubation.
2. PE 10 Intramedic polyethylene tubing (Becton Dickinson, Sparks, MD) for blind instillation of vector into the distal lung.

2.2. Rabbit Procedures

2.2.1. Chemicals

1. Ketamine/xylazine/acepromazine solution for anesthesia.
2. Nair (Carter-Wallace, Somerset, NJ) for hair removal.
3. Omnipaque (300 mgI/mL; Novaplus), a water soluble radio-opaque dye used to document localization of instilled vector by X-ray.

4. EGTA (Fisher Scientific, cat. no. S311-500) for vector formulation.
5. Pentobarbital sodium solution, 300 mg/mL, for euthanasia.

2.2.2. Supplies

1. Laryngoscope (Welch Allyn, Skaneateles Falls, NY) with a #0 blade for intubation.
2. 2.5- and 3.0-mm uncuffed endotracheal tubes (cat. no. 86223; Mallinckrodt, St. Louis, MO).
3. 5 Fr. radio-opaque infant feeding tube (cat. no. 85771; Mallinckrodt).
4. High Temp cauterizing instrument (Accu-Temp, Medtronic Opthalmics, Jacksonville, FL).
5. PE 330 Intramedic polyethylene tubing (Clay Adams) for tracheal cannulation.

3. Methods

3.1. In Vivo Gene Transfer to Mouse Airway Epithelia

Adult mice of approx 6 wk of age are used. Lentiviral vectors can be administered by the nasal, oro-tracheal, or transtracheal routes. We previously found that the receptors for many retroviral envelope pseudotypes including VSV-G, xenotropic, amphotropic, RD114, 10A1, and gibbon ape leukemia virus (GALV) are functionally inaccessible from the apical surface of polarized human airway epithelia (***7***,***27***, and unpublished observations). We subsequently determined that transiently disrupting the epithelial junction complex using vectors formulated in calcium chelators such as EGTA allows for successful gene transfer from the apical surface of airway epithelia in vitro and in vivo ***(7***,***27***,***28)*** (*see* **Note 1**).

3.1.1. Nasal Instillation of Viral Vector

1. Lightly anesthetize the mouse with halothane by placing the animal in an airtight container with a gauze pad soaked in approx 2–5 mL of halothane. The time it takes to anesthetize will vary, but wait until the respiratory rate decreases, the tidal volume increases, and "whisker twitching" slows. Take caution not to wait too long to remove the mouse from the chamber to avoid anesthetic overdose.
2. FIV vector with a β-galactosidase reporter is prepared as described previously ***(7***,***26)***. Apply 50 µL (~10^7 transducing units) of viral vector liquid dropwise to the nares. The vector suspension should be rapidly aspirated into the lungs. To facilitate liquid inhalation, gently hold the mouth closed during instillation.
3. As a control, a radio-opaque solution can be instilled with its presence in the airways confirmed by X-ray **(Fig. 1)**.
4. The mice should recover quickly (<5 min) from the anesthesia.
5. An additional 50 µL of vector may be instilled the following day or at other intervals.
6. After a suitable time interval following vector instillation, euthanize the animal and proceed to detect the reporter gene expression or perform other studies (typically >2 wk). For the X-Gal staining protocol, see **Subheading 3.2.3.**

3.1.2. Oro-Tracheal Administration of Vector

1. Mice are anesthetized using halothane or mixture of ketamine/xylazine/acepromazine.
2. The animal is gently restrained in the supine position and the tongue is retracted anteriorly to visualize the glottis. A 22- or 24-gauge plastic IV catheter is introduced into the trachea. The vector is then administered via the catheter in 50–100 μL volumes.

3.1.3. Transtracheal Vector Administration

To administer vector by the transtracheal route, the mouse is anesthetized as described in **Subheading 3.1.1.** With the animal restrained in the supine position, the ventral neck is disinfected with betadine, followed by 70% alcohol, and a midline incision is made to surgically expose the trachea.

1. Following minimal blunt dissection to expose the larynx and cartilage rings, vector is directly instilled into the proximal trachea between the larynx and first cartilaginous tracheal ring using a tuberculin syringe and needle. Alternatively, PE 10 polyethylene tubing can be threaded into a distal airway via a tracheotomy made at the same position. This approach allows a smaller volume of vector (10–50 μL) to be delivered blindly to the distal lung. The surgical incision is closed in layers and triple antibiotic ointment applied.
2. Following the desired experimental interval, the mouse is euthanized with pentobarbital sodium (300 mg/kg IP), and the tissues are prepared for fixation and staining as required.

3.2. In Vivo Gene Transfer to Rabbit Airway Epithelia

3.2.1. Lower Airway Vector Delivery by Catheter

1. Adult New Zealand white rabbits of approximately 4–6 lbs body weight are used. The animal is anesthetized with 32 mg/kg ketamine, 5.1 mg/kg xylazine, and 0.8 mg/kg acepromazine intramuscularly. Anesthesia usually lasts for over 1 h.
2. The rabbit is placed in the supine position, and the head and neck are extended.
3. A laryngoscope with a #0 blade is placed over the tongue and gentle anterior pressure exerted to visualize the epiglottis and larynx (*see* **Note 2**).
4. Intubation is performed using a 2.5–3.0-mm uncuffed tube.
5. A 5 Fr. radio-opaque infant feeding tube is then passed through the endotracheal tube and slowly advanced into the trachea and bronchi until resistance is felt. This procedure will wedge the tubing in a distal bronchus, usually in the right lung. The position of the feeding tube in the lung can be confirmed by X-ray **(Fig. 2)** with or without instillation of radio-opaque dye.
6. Following X-ray documentation, 0.5–1 mL of FIV vector is introduced into the airways via the tubing using a 5-mL syringe attached to the feeding tube or to a 25-gauge blunt needle for the PE tubing. The volume of liquid is subsequently "pushed" to the lower airways with the slow delivery of 2–4 cc of air.

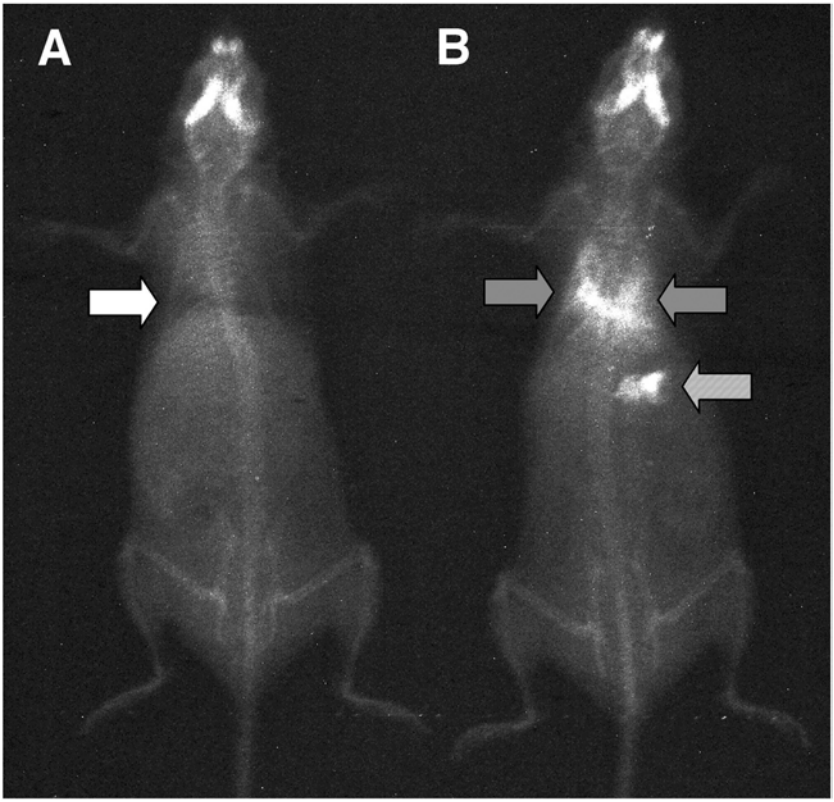

Fig. 1. Radiographic confirmation of delivery of vector solution to the airways following nasal instillation. C57Bl/6 mice were lightly anesthetized with halothane. One-tenth milliliter of the radio-opaque solution Omnipaque was applied to the nares dropwise and allowed to be inhaled. Following recovery, a radiograph was obtained. **(A)** Control mouse with no instillation. **(B)** Test mouse with Omnipaque instilled. The open arrow indicates the clear lung fields in a control mouse. The gray arrows indicate the presence of radiopaque material in the airways of a test mouse. The striped arrow indicates the presence of radiopaque material in the stomach of a test mouse.

7. The infant feeding tubing and the endotracheal tube are then withdrawn and the animal is allowed to recover.
8. After a suitable time period following vector delivery, the animal is euthanized to initiate protocols to detect the reporter gene expression (typically >2 wk). For the X-Gal staining protocol, *see* **Subheading 3.2.3.** (*also see* **Note 3**). An example of results obtained using this approach with a VSV-G-pseudotyped FIV vector expressing cytoplasmic β-galactosidase is shown in **Fig. 3**. In this example, the vector was formulated with EGTA to transiently disrupt epithelial tight junctions.

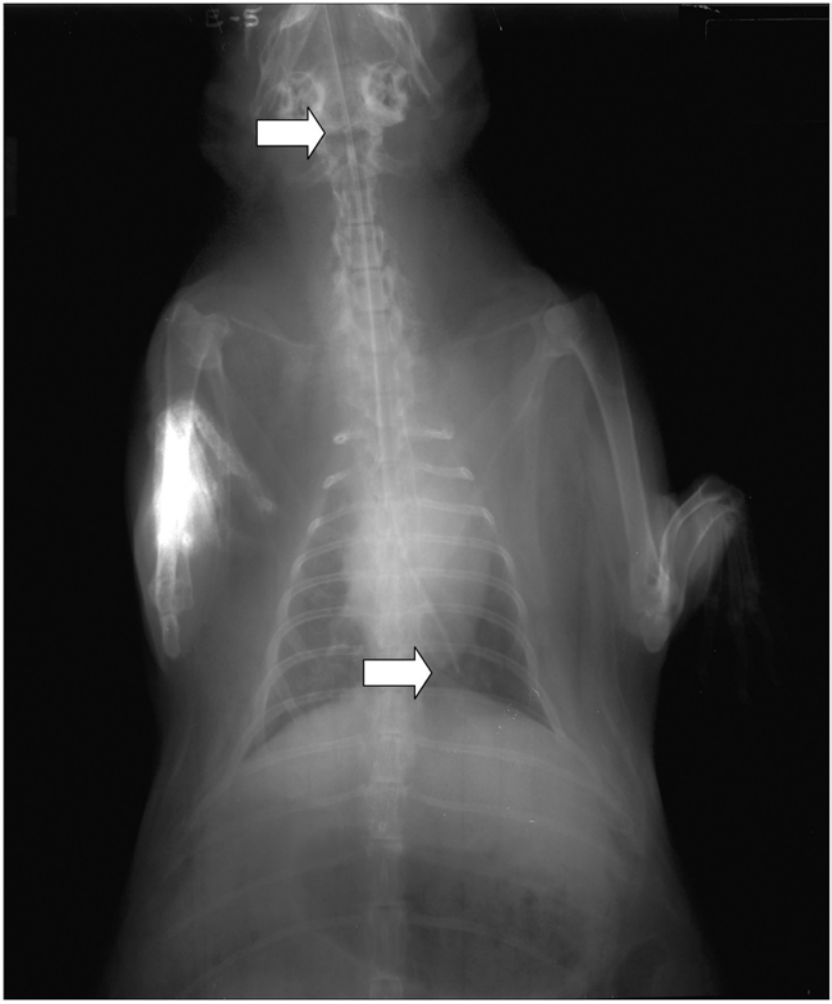

Fig. 2. Chest radiograph confirmation of 5F feeding tube position in rabbit lower airways. The X-ray image localizes the radiopaque infant feeding tube in a peripheral airway of the left caudal lobe prior to vector instillation. Arrows indicate the position of the feeding tube.

3.2.2. Vector Delivery to Tracheal Epithelia by Tracheotomy

1. Adult New Zealand white rabbits of approx 4–6 lbs body weight are used. Animals are anesthetized with 32 mg/kg ketamine, 5.1 mg/kg xylazine, and 0.8 mg/kg acepromazine intramuscularly. Anesthesia usually lasts for over 1 h. If additional anesthesia is required, a half dose can be readministered.

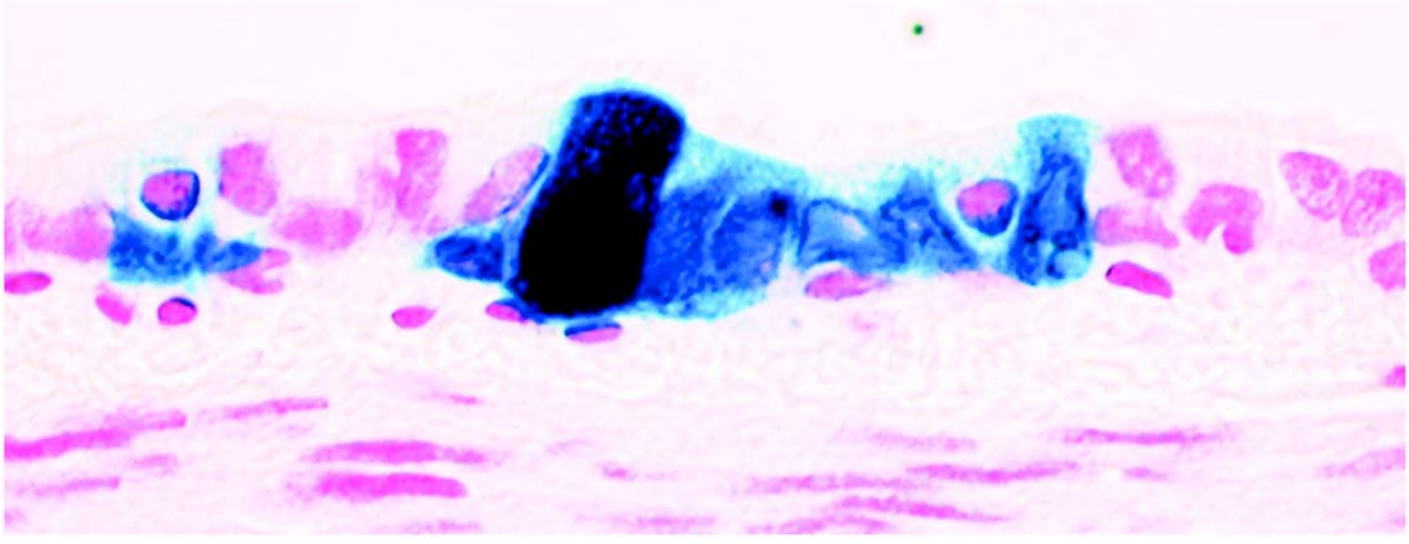

Fig. 3. Gene transfer to rabbit airway epithelia. EGTA-formulated VSV-G-pseudotyped FIV vector expressing β-galactosidase was delivered to the rabbit lower airway via a catheter as described. Five days later gene expression was evaluated using X-Gal staining. Shown are bronchial epithelia expressing β-galactosidase. Note that cells at both the airway surface and basal level were transduced.

2. The rabbit is gently restrained in the supine position. Hair on the ventral neck is removed using a combination of Nair and electric hair clippers.
3. The surgical area is prepared using betadine followed by 70% alcohol.
4. Using standard sterile technique, a ventral midline incision is made and a segment of 1.5- to 2.0-cm length of trachea is exposed and isolated by blunt dissection. A tracheotomy is performed using a High Temp cauterizing instrument. Three tracheotomy incisions are made between the tracheal rings; the most caudal one is used for ventilation. The two cephalad incisions are each cannulated with PE 330 polyethylene tubing and secured in position with suture. This isolates an approx 1.5-cm tracheal segment for the gene transfer experiment.
5. The isolated tracheal segment is rinsed with saline and filled with ~300 μL FIV-β-galactosidase vector in 6 m*M* EGTA in 20 m*M* HEPES buffer (pH 7.2–7.4) for 45 min.
6. The cannulae are removed and tracheal incisions are closed with 4-0 chromic suture. Muscles and superficial fascia are sutured with a 3-0 black monofilament nylon suture. The skin is closed using 0 Vicryl violet braided suture. A tissue adhesive and antibiotic ointment are also applied.
7. Following the desired experimental interval, the animal is euthanized with pentobarbital sodium, and the tissues are prepared for fixation (*see* **Note 4**) and staining.

3.2.3. X-Gal Staining

1. Euthanize the animal and immediately perfuse through the right ventricle with 1X phosphate-buffered saline (PBS) followed by 2% paraformaldehyde.
2. Excise the lungs and the trachea to the larynx (it is not necessary to separate the heart); inflate with 2% paraformaldehyde through the trachea with a syringe and a 22-gauge needle.
3. Submerse in additional 2% paraformaldehyde and allow the tissue to fix at 4°C for 4 h or overnight for larger animals.
4. Following fixation, rinse the lungs with 1X PBS 3 times and inflate with X-Gal solution (*see* **Table 1**).
5. The lungs are then submersed in additional X-Gal solution and incubated overnight at 37°C. Following X-Gal staining, tissues are rinsed three times with PBS before embedding and sectioning.
6. Prepare for sectioning.

4. Notes

1. To formulate FIV-based vectors with EGTA, the stock vector is diluted 1:1 (vol/vol) with 12 m*M* EGTA buffered in 20 m*M* N-2-hydroxyl-ethylpiperazine-N-2-ethanesulfonic acid (HEPES)/HCl (pH 7.4) to attain a final EGTA concentration of 6 m*M*. The final buffered EGTA solution is also hypotonic (osmolality, ~40 mmol/kg). We have successfully used EGTA concentrations from 1.5 to 12 m*M* for in vitro and in vivo applications ***(7,27,28)***. Other calcium chelators such as ethylenediaminetetraacetic acid (EDTA) and BAPTA produce similar

results ***(28)***. The use of calcium chelators and other agents to transiently disrupt tight junctions has been reported for nonretroviral vectors as well ***(29–31)***.

2. Oro-tracheal intubation of rabbits is challenging due to the small oropharynx, large incisors, and the anterior position of the larynx. This procedure can be facilitated by modifying the end of the larynogoscope blade to make its distal width narrower and compatible with rabbit anatomy. Inserting the blade lateral to the central incisors aids in visualization of the larynx.
3. Minimizing background staining with X-Gal: The use of nuclear targeted β-galactosidase transgene constructs facilitates easy identification of true gene transfer and is preferred when possible. Lung tissues have varying degrees of endogenous β-galactosidase activity. This endogenous activity is increased when the pH of the X-Gal staining solution falls below 7.4 and with prolonged incubations at 37°C. Background endogenous activity tends to appear as grainy cytoplasmic staining.
4. Lung tissue often remains buoyant during rinse steps and incubations despite inflation with liquid. To counteract this tendency, use a hot plate to melt beeswax (Fisher) in the bottom of a small glass beaker or scintillation vial and allow to cool. Secure the lung to the bottom of the beaker by pushing an 18-gauge needle through the heart and into the wax.

Acknowledgments

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References

1. Hacein-Bey-Abina, S., Le Deist, F., Carlier, F., et al. (2002) Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N. Engl. J. Med.* **346,** 1185–1193.
2. Welsh, M. J. (1999) Gene transfer for cystic fibrosis. *J. Clin. Invest.* **104,** 1165–1166.
3. McCray, P. B., Jr. (2001) Difficulties of gene therapy. *Lancet* **358 (Suppl.),** 19.
4. Poeschla, E. M., W-Staal, F., and Looney, D. L. (1998) Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. *Nature Med.* **4,** 354–357.
5. Johnston, J. C., Gasmi, M., Lim, L. E., et al. (1999) Minimum requirements for efficient transduction of dividing and nondividing cells by feline immunodeficiency virus vectors. *J. Virol.* **73,** 4991–5000.

6. Naldini, L., Blomer, U., Gallay, P., et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.
7. Wang, G., Slepushkin, V. A., Zabner, J., et al. (1999) Feline immunodeficiency virus vectors persistently transduce nondividing airway epithelia and correct the cystic fibrosis defect. *J. Clin. Invest.* **104,** R49–R56.
8. Goldman, M. J., Lee, P.-S., Yang, J.-S., and Wilson, J. M. (1997) Lentiviral vectors for gene therapy of cystic fibrosis. *Hum. Gene Ther.* **8,** 2261–2268.
9. Shami, S. G. and Evans, M. J. (1991) Kinetics of pulmonary cells, in *Comparative Biology of the Normal Lung* (Parent, R. A., ed.), CRC Press, Boca Raton, FL, pp. 145–155.
10. Johnson, L., Olsen, J., Naldin, L., and Boucher, R. (2000) Pseudotypes human lentiviral vector-mediated gene transfer to airway epithelia in vivo. *Gene Ther.* **7,** 568–574.
11. McCormack, J. E., Martineau, D., DePolo, N., et al. (1997) Anti-vector immunoglobulin induced by retroviral vectors. *Hum. Gene Ther.* **8,** 1263–1273.
12. Hartmann, K. (1998) Review feline immunodeficiency virus infection: an overview. *The Veterinary J.* **155,** 123–137.
13. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J.-K. (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: Concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* **90,** 8033–8037.
14. Evans, M. J., Johnson, L. V., Stephens, R. J., and Freeman, G. (1976) Renewal of the terminal bronchiolar epithelium in the rat following exposure to NO_2 or O_3. *Lab. Invest.* **35,** 246–257.
15. Plopper, C. G., Nishio, S. J., Kass, A. P., and Hyde, D. M. (1992) The role of the nonciliated bronchiolar epithelial (Clara) cell as the progenitor cell during bronchiolar epithelial differentiation in the perinatal rabbit lung. *Am. J. Respir. Cell Mol. Biol.* **7,** 606–613.
16. Johnson, N. F. and Hubbs, A. F. (1990) Epithelial progenitor cells in the rat trachea. *Am. J. Respir. Cell Mol. Biol.* **3,** 579–585.
17. Inayama, Y., Hook, G. E. R., Brody, A. R., et al. (1988) The differentiation potential of tracheal basal cells. *Lab. Invest.* **58,** 706–717.
18. Rai, S. K., DeMartini, J. C., and Miller, A. D. (2000) Retrovirus vectors bearing jaagsiekte sheep retrovirus Env transduce human cells by using a new receptor localized to chromosome 3p21.3. *J. Virol.* **74,** 4698–4704.
19. Kobinger, G. P., Weiner, D. J., Yu, Q. C., and Wilson, J. M. (2001) Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. *Nat. Biotechnol.* **19,** 225–230.
20. Wang, G., Deering, C., Macke, M., et al. (2000) Human coronavirus 229E infects polarized airway epithelia from the apical surface. *J. Virol.* **74,** 9234–9239.
21. Wang, G., Sinn, P. L., and McCray, P. B., Jr. (2000) Development of retroviral vectors for gene transfer to airway epithelia. *Curr. Opin. Mol. Ther.* **2,** 497–506.

22. Sinn, P. L., Williams, G., Vongpunsawad, S., Cattaneo, R., and McCray, P. B., Jr. (2002) Measles virus preferentially transduces the basolateral surface of well-differentiated human airway epithelia. *J. Virol.* **76,** 2403–2409.
23. Bayle, J., Johnson, L. G., St. George, J. A., Boucher, R. C., and Olsen, J. C. (1993) High-efficiency gene transfer to primary monkey airway epithelial cells with retrovirus vectors using the gibbon ape leukemia virus receptor. *Hum. Gene Ther.* **4,** 161–170.
24. Goff, S. P. (2001) Intracellular trafficking of retroviral genomes during the early phase of infection: viral exploitation of cellular pathways. *J. Gene Med.* **3,** 517–528.
25. Wang, G., Williams, G., Xia, H., et al. (2002) Apical barriers to airway epithelial cell gene transfer with amphotropic retroviral vectors. *Gene Ther.* **9,** 922–931.
26. Stein, C. S., Kang, Y., Sauter, S. L., et al. (2001) In vivo treatment of hemophilia A and mucopolysaccharidosis type VII using nonprimate lintiviral vectors. *Mol. Ther.* **3,** 850–856.
27. Wang, G., Davidson, B. L., Melchert, P., et al. (1998) Influence of cell polarity on retrovirus-mediated gene transfer to differentiated human airway epithelia. *J. Virol.* **72,** 9818–9826.
28. Wang, G., Zabner, J., Deering, C., et al. (2000) Increasing epithelial junction permeability enhances gene transfer to airway epithelia in vivo. *Am. J. Respir. Cell Mol. Biol.* **22,** 129–138.
29. Walters, R. W., Grunst, T., Bergelson, J. M., Finberg, R. W., Welsh, M. J., and Zabner, J. (1999) Basolateral localization of fiber receptors limits adenovirus infection of airway epithelia. *J. Biol. Chem.* **274,** 10,219–10,226.
30. Duan, D., Yue, Y., McCray, P. B., Jr., and Engelhardt, J. F. (1998) Polarity influences the efficiency of recombinant adeno-associated virus infection in differential airway epithelia. *Hum. Gene Ther.* **9,** 2761–2776.
31. Coyne, C. B., Kelly, M. M., Boucher, R. C., and Johnson, L. G. (2000) Enhanced epithelial gene transfer by modulation of tight junctions with sodium caprate. *Am. J. Respir. Cell Mol. Biol.* **23,** 602–609.

23

Cells of the Nervous System

Carl Rosenblad and Cecilia Lundberg

1. Introduction

Direct in vivo gene transfer to the central nervous system (CNS) using recombinant lentiviral vectors (rLV) has emerged as a powerful technique to overexpress various genes of interest in different neuronal populations. This interest is exemplified by the increasing number of studies using rLV vectors to evaluate therapeutic proteins to correct disorders of the nervous system ***(1–3)*** or to explore a protein's involvement in normal function or pathological processes ***(4)***. Compared to conventional trangenic techniques for overexpression, rLVs have several attractive features. For example, the level of transgene expression in cells transduced with a rLV is typically higher than obtained with knock-in techniques and is present already at 3–4 d after injection in vivo ***(5)***. Second, the expression may be directed to specific regions or cell populations depending on the anatomical location of the rLV injections as well as the design of the vector system. This may be advantageous for the study design since it allows unilateral overexpression, thereby creating an internal control in functional and morphological studies. Finally, it is considerably less time consuming than creating transgenic mouse lines.

There are today a number of rLV systems available, and the characteristics and potential advantages/disadvantages of the respective system have been described in other chapters in this book. The ability to transduce nondividing CNS cells in vivo have been reported for recombinant systems derived from human immunodeficiency virus (HIV) ***(6,7)***, equine infectious anemia virus (EIAV) ***(8)***, and feline immunodeficiency virus (FIV) ***(9)***.

Here we describe a method for intracerebral injection of a HIV type 1 (HIV-1)-derived third generation vesicular stomatitis virus G glycoprotein (VSV-G)-

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pseudotyped self-inactivating (SIN)-rLV vector ***(10)*** in the adult rat CNS. The vector described contains green fluorescent protein (GFP) as transgene since this is one of the most commonly used rLVs for transduction in the nervous system. GFP gets distributed throughout the cell body and neuritic tree of transduced cells and has in a number of studies been found to allow detailed morphological evaluation of transduced cells in tissue sections ***(5,11)***. Using fluorescence microscopy, the GFP can be directly visualised in the sections without further histochemical processing. Because of this visibility and the stability of the protein, it can also easily be combined with other immunohistochemical/immunofluorescence techniques. Although direct visualization of GFP-expressing cells may be sufficient in most cases, increased detection sensitivity may be desirable in cases where transgene expression is low or the details of the morphology have to be further enhanced. This can be obtained by immunohistochemical amplification using antibodies to GFP as described in **Subheadings 2.** and **3.** below. We use perfusion fixation and free-floating processing of the tissue sections since this has been found to give very good morphological quality compared to immersion fixation or snap frozen preparations, but the protocol outlined may still be used for transgene detection in all preparations. The protocol outlined has also been used for detection of several other transgenes, such as glial cell line-derived neurotrophic factor (GDNF) ***(5)*** and β-galactosidase (lacZ) ***(2)***, following rLV transduction and staining with specific antibodies.

The vector described containing the cytomegalovirus (CMV) promoter has been found to transduce both neurons, glia, and oligodenrocytes in several different structures of the nervous system ***(3,6)***, including striatal, hippocampal, septal, retinal, cortical, and midbrain cells ***(1–3,6,12–14)***. However, depending on the specific paradigm in which it is to be used, a vector with a somewhat different transduction and expression pattern may be advantageous. It has been shown that the internal promoter used to drive transgene expression ***(12–14)***, as well as the envelope used ***(15)***, influences where and how strongly the transgene is expressed. The infection rate in the rat and monkey CNS have been estimated to be around 1 infected cell per 40 injected transducing units (TU) (MOI [multiplicity of infection] 40) and appears to be linear at least over a range ***(5)***, but transduction efficiency of up to 1 transduced cell per 10 TU or less have been reported ***(16)***.

Although several investigators have found SIN rLV vectors to be completely free of wild-type particles ***(10,17–19)*** an important aspect of direct in vivo gene transfer using lentiviral vectors is to ensure safe and appropriate handling of the vector. Although safety related to production of virus has been covered in earlier chapters of this book, the importance of good working routines should

be emphasized. Exactly what these routines are is dependent on vector type, vector construct, and transgene, as well as on local rules, but in general a P2 biosafety level laboratory is preferred (*see* **Note 1**). The routines should ideally contain a "safety margin" rather than a minimal safety level. This will help minimize the risk that people working with the vectors will be accidentally infected and also avoid work with HIV-based vectors that might alarm people working in the same facilities, but not directly with the virus, thereby avoid creating an unnecessary fear of this technology. The procedure described below is carried out in a P2 facility.

2. Materials

2.1. Intracerebral Injection

2.1.1. Safety, Protection

1. Biosafety Level 2 laboratory (*see* **Note 1**), ventilated bench.
2. 3% hydrogen peroxide.
3. Antiviral detergent solution (1% Virkon S, Antec International, UK).
4. Protective clothing (coat, disposable gloves, protective eyewear).
5. Log book in which details about surgery and virus are given (to be kept in the surgery facility).
6. Animal facility for 24–48 h postsurgical quarantine.

2.1.2. Surgical Equipment

1. Gas or injection anaesthetics to induce general anaesthesia in small animals: halothane (Halocarbon Laboratories, River Edge, NJ), isoflurane (Baxter, Deerfield, IL), or sodiumpentobarbital. Gas anesthesia requires access to air or technical gases (O_2 + N_2O) and mixer (Penlon, Sigma, Penlon, Abingdon, UK).
2. Small animal stereotaxic frame (Stoelting Lab Standard, cat. no. 51600, equipped with large probe holder, cat. no. 51633, and if applicable, gas anesthesia mask, cat. no. 51610; Stoelting, Wood Dale, IL).
3. Surgical microscope (Leica M715; Leica Microsystems, Wetzlar, Germany).
4. Bone drill.
5. 2–10 μL Hamilton Microliter syringe with 26-gauge steel cannula. Optional: For more precise injections with less tissue damage, a microinjection technique can be used as described by Nikkah et al. ***(19)*** (*see* also **Fig. 1**). This requires in addition to the Hamilton syringe, a pulled glass pipet (Drummond Microcaps 50 μL with an outer diameter of 50–100 μm at the tip; Drummond Scientific, Broomall, PA), plastic tubing (polyethylene tubing, Intramedic O.D., 0.965 mm, I.D., 0.58 mm), 25-gauge 1.5-in. needle.
6. Suture material, such as Ethicon 3/0 suture kit (Ethicon, Cornelia, GA), or metal clips (Agraf Michel, 7.5 × 1.75 mm, Martin, Germany) and a clip-forceps (Martin, Jacksonville, FL).
7. Surgical instruments (scalpel, peang, microforceps).

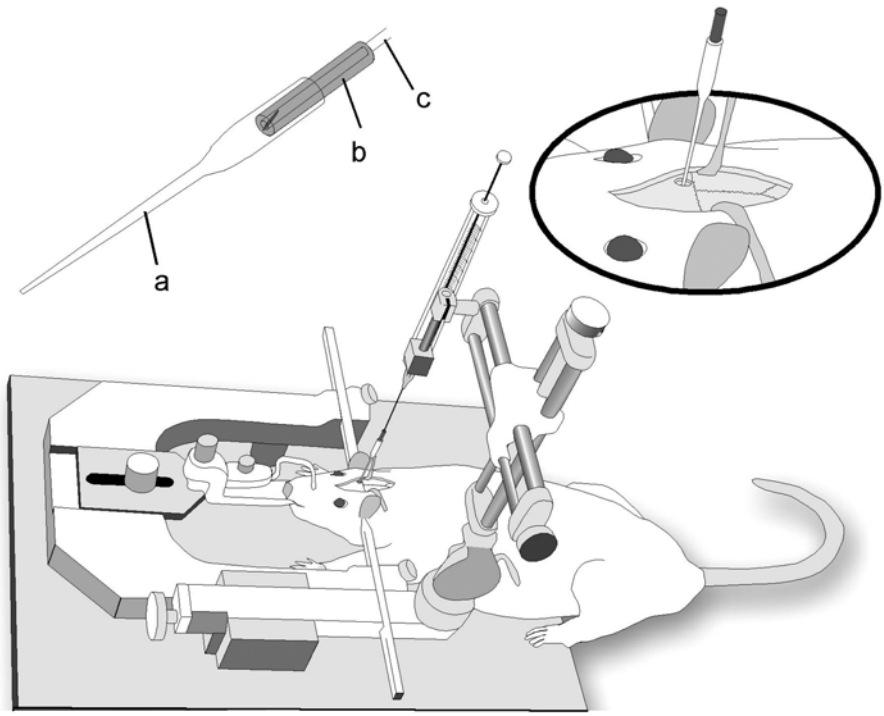

Fig. 1. Illustration of the general set up for an intracerebral injection as described in this chapter. The anaesthetized rat is fixed in the stereotaxic frame, and the Hamilton injection needle (adapted for the microinjection technique as shown in the upper left corner) is placed vertically. The high magnification of the microinjection assembly show how a piece of plastic tubing **(b)** is put on the Hamilton needle **(a)** to avoid leakage between the glass capillary **(c)** and the Hamilton needle. The encircled magnification in the upper left corner schematically illustrates the view of an injection cannula in position for an injection into the right hemisfere slightly anterior to the bregma (typically the head of the caudate-putamen; *see* **Note 3**).

2.1.3. Viral Preparation

1. Concentrated viral stock containing preferably $>1 \times 10^8$ TU/mL, should be stored at –80°C until use in order to preserve viral titer.
2. Media for dilution of viral stock. To decrease the risk of accidental aerosols of viral suspension, a maximum viral working preparation volume of 100 μL/vial should be used (*see* **Note 1**).

2.2. Tissue Preparation

1. 4% w/v paraformaldehyde (PFA), pH 7.0 to 7.4 at 4°C.
2. 0.9% w/v NaCl in deionized water, or phosphate-buffered saline (PBS), room temperature.

3. 2 peang, 1 pair of scissors.
4. 20–30% w/v sucrose in deionized water.
5. 2 mm O.D. prefusion cannula connected via synthetic rubber tubing to a pump adjustable to a flow of 1–3 L/h.
6. Cryostat (Leica, CM3050) or freezing microtome (Leica, SM 2000R).

2.3. Immunohistochemical Transgene Detection

1. 20-mL glass or plastic vials.
2. Quenching solution: 10% v/v methanol + 3% v/v hydrogen peroxide in PBS.
3. Incubation buffer: 2% v/v normal serum (Rockland, Inc., Gilbertsville, PA, cat. no. D109-00) + 0.25% v/v Triton X-100 (Sigma, St. Louis, MO) in PBS.
4. Primary anti-GFP antibody (chicken-anti-GFP, Chemicon, Temecula, CA, cat. no. AB 16901).
5. Secondary antibody (biotinylated rabbit-anti-chicken, Promega, cat. no. G2891).
6. Vectastain ABC kit (Elite PK-6101, Vector Laboratories, Burlingame, CA).
7. Color reaction: 3′3′-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO); 3% hydrogen peroxide.
8. 99.5% ethanol.
9. Xylene.
10. DPX Mountant (Sigma-Aldrich).
11. Glass slides, chromalun coated or positively charged (SuperFrost Plus, Menzel-Glässer, Germany).
12. Cover slips.

3. Methods

3.1. Intracerebral Injection

1. Note date and vector information in the log book to be kept in the surgery room.
2. Take out viral aliquot from freezer and thaw. If necessary, add buffer to obtain desired virus concentration (*see* **Note 2**). Mix the viral preparation by gently pipetting the solution a few times and keep on ice. Gentle mixing of the viral prep may be repeated occasionally to maintain even particle concentration throughout the surgery session.
3. Anaesthetize the animal using a 30% O_2 + 70% N_2O mix containing 1.5 to 2% isoflurane (or halothane) or inject anaesthetics according to manufacturer's instructions.
4. Place anaesthetised animal in the stereotaxic frame as shown in **Fig. 1** and make an incision in the skin over the skull. Remove connective tissue so that the bregma (*see* **Note 3** and **Fig. 1**) is clearly visible and drill a hole in the skull bone at the appropriate anteroposterior and mediolateral coordinates relative to bregma. Make sure that the needle does not touch the bone edges, thereby deviating from the intended trajectory.
5. Load virus into syringe. Lower the tip of the needle until it touches the dura and then read the dorso-ventral coordinate. Lower the needle to the appropriate

dorso-ventral coordinate. If the microinjection method is applied (*see* **Note 4**), a small incision in the dura should be made with the tip of a syringe in order to avoid breaking the tip when penetrating the dura. Inject approx 0.5 µL/min and wait 3 min before retracting needle (*see* **Note 5**).
6. Suture/clip skin incision.
7. Throw all disposables, including remaining viral-containing solutions, in a separate container. All material should then be autoclaved.
8. Clean all nondisposable equipment and table surfaces thoroughly with Virkon S (detergent) or ethanol.
9. Animals should be put in quarantine for 24 to 48 h in order for any viruses in the fur or wound to become inactivated.

3.2. Tissue Preparation

Best morphological quality is obtained from fixed material but other tissue preparation methods may be used as well. Details of transcardial perfusion can be found in **ref. *20***.

1. For perfusion fixation of rat brain, close the descending aorta with a peang and perfuse with 50 mL room temperature saline for 1 to 2 min, followed by 200 mL ice cold 4% PFA for 5–6 min. Dissect out the brain and post-fix in the same fixative for 4 to 6 h. Transfer into 20–30% sucrose and store at 4°C until the brain has sunk (~24 h) before cutting.
2. Cut 12- to 40-µm-thick sections on freezing microtome or cryostat (*see* **Note 6**).

3.3. Immunohistochemical Transgene Detection

Sections containing GFP-transduced cells can be inspected directly using fluorescence microscopy (*see* **Note 7**), but generally the morphological quality is better if immunohistochemistry is used. All incubations in **steps 1–5** are made in 20-mL glass or plastic vials placed on a shaker. Rinses and quenching are made in 2 to 4 mL, and all incubations (primary, secondary, ABC, and DAB) in 1 mL of solution, respectively.

1. Quench sections in quenching solution for 5 min, followed by 3 × 5 min rinses in PBS.
2. Preincubate sections shaking in incubation buffer for 1 h.
3. Incubate with primary antibody (usually 1:1000 to 3000) in incubation buffer for 16 to 18 h at room temperature.
4. Rinse 3 × 5 min with PBS.
5. Incubate with secondary antibody (usually 1:200 to 400) in incubation buffer for 2 h.
6. Rinse 3 × 10 min in PBS.
7. Incubate with ABC according to manufacturer's instructions (half the recommended amount is sufficient for most stainings).

8. Rinse 3 × 10 min in PBS.
9. Color reaction: Mix DAB solution according to manufacturer's instructions. Add 1 mL of DAB solution to the sections and then 10 μL of 3% hydrogen peroxide. The color reaction should be developed in 1 to 2 min. Stop reaction by rinsing 3X in PBS.
10. Mount sections onto glass slides and let air dry overnight.
11. Dehydrate in increasing concentrations of ethanol (70, 95, and 99.5%) for 3 min each and then in xylene 2 × 3 min.
12. Add a few drops of DPX Mountant on the glass slide and cover slip.

4. Notes

1. One of the most likely accidents when handling viral suspensions is a spill. To minimize the risk of potential aerosols, we use small volumes of virus (<100 μL/vial). For more information on safety guidelines and protocols the following websites are available: European Commission directives at http://europa.eu.int/eurlex/en/lif/dat/2000/en_300D0608.html and the Centers for Disease Control (CDC) and National Institute of Health (NIH)'s Biosafety in Microbiological and Biomedical Laboratories at http://bmbl.od.nih.gov/.
2. We have noticed that in order to obtain good transduction in vivo a titer of 1×10^8 TU/mL or above is desirable. This titer will allow a small volume to be deposited in a precise location and thus create a high local MOI that efficiently transduce the cells.
3. Bregma is the T-crossing in the bone sutures between the parietal and the frontal bones and is used as the "anchor-point" for the rostro-caudal and medio-lateral coordinates ***(21)***.
4. Pull a glass pipet so that the outer diameter is 50–100 μm in diameter. Put a piece of polyethylene tubing onto the 26-gauge Hamilton steel needle. Then, put the glass capillary on. Remove the plunger from the syringe and flush the system with saline. Make sure that there is good flow through the capillary and no leakage between the steel cannula and the glass capillary **(Fig. 1)**. Fill the Hamilton syringe and attached glass capillary entirely with saline. Pull up 1 μL of air and then the volume of viral suspension to be injected. The air bubble created in this way will prevent the viral suspension from mixing with the vehicle in the needle and will also serve as a visual cue that the viral suspension is injected. Lower the tip of the glass capillary to the dura and cut the dura below it with the tip of an injection needle so that the glass capillary does not break.
5. The in vivo transduction is generally more confined when a small volume is injected and the needle is left in place for a few minutes to avoid backflow up along the needle track.
6. When cutting sections to be processed free floating for immunohistochemistry according to the protocol outlined here, it is not recommended to go less than 30 μm in thickness. Thinner sections can be used when they are directly mounted onto glass slides and then stained.

7. For direct fluorescence microscopy of GFP+ cells, mount sections onto glass slides, coverslip with fluorescence mounting medium, and view in the microscope under epi-illuminesence (excitation wavelength 455 nm). GFP-positive cells have a clear bright green color not to be confused with the more yellow-green autofluorescence that can be observed in macrophages and other blood cells that are found near the injection site.

References

1. de Almeida, L. P., Zala, D., Aebischer, P., and Deglon, N. (2001) Neuroprotective effect of a CNTF-expressing lentiviral vector in the quinolinic acid rat model of Huntington's disease. *Neurobiol. Dis.* **8,** 433–446.
2. Kordower, J. H., Emborg, M. E., Bloch, J., et al. (2000) Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* **290,** 767–773.
3. Rosenblad, C., Gronborg, M., Hansen, C., et al. (2000) In vivo protection of nigral dopamine neurons by lentiviral gene transfer of the novel GDNF-family member neublastin/artemin. *Mol. Cell Neurosci.* **15,** 199–214.
4. Lobianco, C., Ridet, J. L., Zurn, A., Deglon, N., and Aebischer, P. (2001) Dopaminergic cell loss induced by lentiviral mediated overexpression of wild type or mutant human alpha synuclein in the substantia nigra of rats. *Society for Neuroscience Abstract* **324,** 10.
5. Georgievska, B., Kirik, D., Rosenblad, C., Lundberg, C., and Bjorklund, A. (2002) Neuroprotection in the rat Parkinson model by intrastriatal GDNF gene transfer using a lentiviral vector. *Neuroreport* **13,** 1–8.
6. Blomer, U., Naldini, L., Kafri, T., Trono, D., Verma, I. M., and Gage, F. H. (1997) Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J. Virol.* **71,** 6641–6649.
7. Naldini, L., Blomer, U., Gage, F. H., Trono, D., Verma, I. M. (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl. Acad. Sci. USA* **93,** 11,382–11,388.
8. Mitrophanous, K., Yoon, S., Rohll, J., et al. (1999) Stable gene transfer to the nervous system using a non-primate lentiviral vector. *Gene Ther.* **6,** 1808–1818.
9. Alisky, J. M., Huges, S. M., Sauter, S. L., et al. (2000) Transduction of murine cerebellar neurons with recombinant FIV and AAV5 vectors. *Neuroreport* **11,** 2669–2673.
10. Zufferey, R., Dull, T., Mandel, R. J., et al. (19??) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* **72,** 9873–9880.
11. Kirik, D., Rosenblad, C., Bjorklund, A., and Mandel, R. J. (2000) Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. *J. Neurosci.* **20,** 4686–4700.
12. Kostic, C., Chiodini, F., Hornfeld, D., et al. (2001) Activity analysis of viral and housekeeping gene promoters using self-inactivating lentivirus vector delivery into the mouse retina. *Society for Neuroscience Abstract* **720,** 8.

13. Ericson, C. A., Jansson, M., Björk, E., Wictorin, K., and Lundberg, C. (2001) In vivo studies of different promoters using a lentiviral vector system. *Society for Neuroscience Abstract* **257,** 17.
14. Miyoshi, H., Takahashi, M., Gage, F. H., and Verma, I. M. (1997) Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. *Proc. Natl. Acad. Sci. USA* **94,** 10,319–10,323.
15. Mazarakis, N. D., Azzouz, M., Rohll, J. B., et al. (2001) Rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery. *Hum. Mol. Genet.* **10,** 2109–2121.
16. Kordower, J. H., Bloch, J., Ma, S. Y., et al. (1999) Lentiviral gene transfer to the nonhuman primate brain. *Exp. Neurol.* **160,** 1–16.
17. Dull, T., Zufferey, R., Kelly, M., et al. (1998) A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* **72,** 8463–8471.
18. Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H., and Verma, I. M. (1998) Development of a self-inactivating lentivirus vector. *J. Virol.* **72,** 8150–8157.
19. Nikkhah, G., Olsson, M., Eberhard, J., Bentlage, C., Cunningham, M. G., and Bjorklund, A. (1994) A microtransplantation approach for cell suspension grafting in the rat Parkinson model: a detailed account of the methodology. *Neuroscience* **63,** 57–72.
20. Jonkers, B. W., Sterk, J. C., and Wonterlood, F. G. (1984) Transcardial perfusion fixation of the CNS by means of a compressed-air-driven device. *J. Neurosci. Meth.* **12,** 141–149.
21. Paxinos, G. and Watson, C. (1997) The rat brain in stereotaxic coordinates, 3rd ed., Academic Press, San Diego, CA.