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ORIGINAL RESEARCH PAPER

Genomic integration occurs in the packaging cell via unexported lentiviral precursors

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Abstract
Objective To use HIV-1 based lentivirus components to produce gene integration and the formation of a stable cell line in the packaging cell line without viral infection.
Results A co-transfection of a Human Embryonic Kidney (HEK) 293 packaging cell line with Gag–pol (GP) and a transfer vector, without the envelope vector, produces a stable cell line after 2 weeks of selection. Furthermore, a matrix protein deficient GP in the packaging vector enhances this integration. This supports that, in theory, unexported lentiviral cores produced within the packaging cell can infect itself without requiring the release of any lentiviral particles.
Conclusion If the packaging cell is also the target cell, then gene integration leading to a stable cell line can be accomplished without viral particle infection.

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Introduction

HIV-1-based lentiviruses have been used to deliver and irreversibly integrate foreign genetic material into target cells, allowing for the generation of stable cell lines (Quinonez and Sutton 2002). The typical protocol for second generation lentiviral production involves a triple transfection of packaging, envelope and transfer vectors into a packaging cell line such as Human Embryonic Kidney (HEK) 293 cells (Hewinson et al. 2013). First, the packaging vector encodes for a polyprotein called Gag–pol (GP), which is cleaved into other proteins such as the matrix (MA), capsid (CA), and integrase (IN) proteins. CA assembles the genetic material into a lentiviral core; MA facilitates viral core budding; IN integrates viral DNA into the target cell's genome (Kaplan 2002). Second, the envelope vector encodes a protein that coats the membrane of the lentivirus to facilitate entry into the target cell via endocytosis. For broad specificity, a common envelope protein is the vesicular stomatitis virus G-protein (VSVG) as it targets the LDL receptor found on many mammalian cells (Finkelshtein et al. 2013). However, due to the toxicity of VSVG, it is difficult to produce a stable packaging cell line for mass production of lentivirus (Burns et al. 1993; Chen

et al. 1996; Zhang et al. 2004). Lastly, the transfer vector encodes the foreign genetic material that is transcribed into lentiviral RNA. Lentivirus bud off from the packaging cell, carrying a piece of the plasma membrane, a cone shaped viral core containing viral RNA as well as other GP-derived proteins that facilitate viral integration. Since mass concentration of lentivirus is a crucial step in standard viral infection of target cells (Ricks et al. 2008), there are often health and safety considerations involved in this process.

While the objective of mass production of lentivirus via packaging cells is to integrate foreign genetic material into target cells, it is plausible that genomic integration occurs in the packaging cell as well. Lentiviral entry involves a two step process: first, attachment of the viral particle to the target cell via the envelope protein and second, entry of the viral core into the target cell via endocytosis (Clapham and McKnight 2002). Once the lentiviral core enters the target cell cytoplasm, it must be uncoated to release its contents, a process thought to involve the ubiquitous peptidylprolyl isomerases (Ambrose and Aiken 2014). While assembled lentiviral cores are being packaged into viral particles, some unexported viral cores may self-infect the packaging cell. Here, to test this possibility, we showed that a co-transfection of a transfer vector and GP produces more DNA integration into HEK293 packaging cells than controls. Furthermore, a co-transfection with a MA deficient GP had the greatest prevalence for integration. In cases where the packaging cell is the target cell, this method of self-infection does not require the production of any lentiviral particles, and therefore has fewer health and safety considerations.

Materials and methods

Vector construct and synthesis

DNA isolation and manipulations were performed according to Davis (2012). The vectors SINp-Cherry, GP, GP (NoMA), and GP (NoIN) were synthesized by Genscript (Pescataway, NY) and subcloned into the pUC57-Simple vector using *Eco*RV. The pMD2.G vector, created by Didier Trono, was ordered through Addgene (plasmid # 12259).

Cell culture and transfection

The cell lines used in this study were HEK 293 (ATCC; CRL-1573), HEK 293T (ATCC; CRL-3216), CHO (ATCC; CRL-11965), and 3T3 cells (ATCC; CCL-92). All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 25 mM D-glucose, 1 mM sodium pyruvate, 4 mM L-glutamine, 10 % (v/v) FBS, and 10 mg penicillin–streptomycin/l in T5 flasks at 37 °C and 5 % CO_2. Cells were grown to 95 % confluency before passaging with 0.05 % trypsin/EDTA. Passaged cells were seeded onto 24-well plastic plates at 1:10 dilution and grown overnight. Cells were transiently transfected with vectors and Lipofectamine 2000 according to manufacturer's protocols (Dalby et al. 2004).

Imaging and illumination

All cell imaging were performed in DMEM media, using an inverted non-confocal microscope with a Lambda DG4 xenon lamp source and Tuscen H674ICE CCD camera. Excitation and emission filter specifications for RFP were 580/20 and 630/60 nm respectively (Semrock). Micro-manager open source microscopy software was used.

Statistical analysis

Data is presented as the mean ± SD with at least three independent experiments with over 200 colonies counted in total. Significance between conditions was calculated using ANOVA and Tukey's HSD test, and $p < 0.05$ was considered statistically significant.

Results and discussion

Co-transfection with Gag-pol and a transfer vector produces gene integration

In a standard lentiviral infection protocol involving a packaging cell and a target cell, viral infection leading to DNA integration in the target cell is the desired outcome. However, DNA integration can also occur in the packaging cell via a mechanism resembling self-infection. To test this possibility we synthesized two vectors: a packaging vector consisting of a cytomegalovirus

promoter (CMVp) preceding HIV-1 Gag-pol (hereafter, GP), and a self inactivating transfer vector (Yu et al. 1986) (hereafter, SINp-Cherry), consisting of a tandem fusion of the plasma membrane localization peptide from Lyn kinase (Violin et al. 2003) with a monomeric red fluorescent protein mCherry (Fink et al. 2010) regulated by CMVp as well as blasticidin resistance regulated by a SV40 promoter (Supplementary Fig. 1a). Although a portion of the mCherry is still detectable in the cytoplasm, the plasma localization peptide Lyn was used to allow for better visualization of mCherry in the boundaries of fluorescent cells such that they can be distinguished within fluorescent colonies.

In a single well of a 24-well plate, a co-transfection of the GP packaging vector and SINp-Cherry transfer vector produced a stable cell line of HEK293 cells, expressing Lyn-mCherry after a 3 week selection period with blasticidin (Fig. 1a, b) ($n = 3$ experiments). There are three possibilities for this outcome. First, since the transfer vector has blasticidin resistance, surviving cells retaining the transfer vector could have been integrated it into the genome during cell division by random integration. Random integration is rarer than targeted integration and often inserts DNA fragments into unstable mammalian loci rendering them non-functional or silenced (Merrihew et al. 1996; Vasquez et al. 2001), hence it is unlikely here given our small-scale transfection (i.e. 24-well plate). Second, exported lentiviral particles produced by GP could have infected adjacent cells. With the absence of envelope proteins such as VSVG, these particles lack a mechanism of entering the cells by endocytosis and hence this is also unlikely. Third, unexported lentiviral particles could have self-infected the transfected cells (as illustrated in Supplementry Fig. 1b).

Lentiviral particles produced by GP are not infective in the absence of the VSVG envelope protein

One possibility for the outcome for the production of stable cell lines from a co-transfection (Fig. 1a, b) is that the GP vector could export lentiviral particles in the absence of an envelope vector that might somehow still infect adjacent cells to produce DNA integration. Although these particles lack a mechanism of entering cells, other forms of entry, although rare, may exist (Mondal Roy and Sarkar 2011). Hence, a protocol of small-scale lentiviral infection was designed to test the infectivity of exported lentiviral particles, where we used HEK293 cells as the packaging cell and HEK293T cells as the target cell (illustrated in Supplementary Fig. 2). The relevant difference is that the HEK293T cell has neomycin resistance, while HEK293 does not (Swift et al. 2001). Packaging cells were co-transfected with GP and SINp-Cherry or triple transfected with GP, SINp-Cherry, and VSVG [i.e. the pMD2.G vector (Nasri et al. 2014)]. The presence of VSVG was assayed via pH induced membrane fusion, which is a well-known characteristic of VSVG (Nagaraj et al. 2013).

In the absence of VSVG, cell fusion did not occur and singular fluorescent cells remain clearly delineated (Fig. 2a, e). In the presence of VSVG, pH-induced membrane fusion with adjacent cells

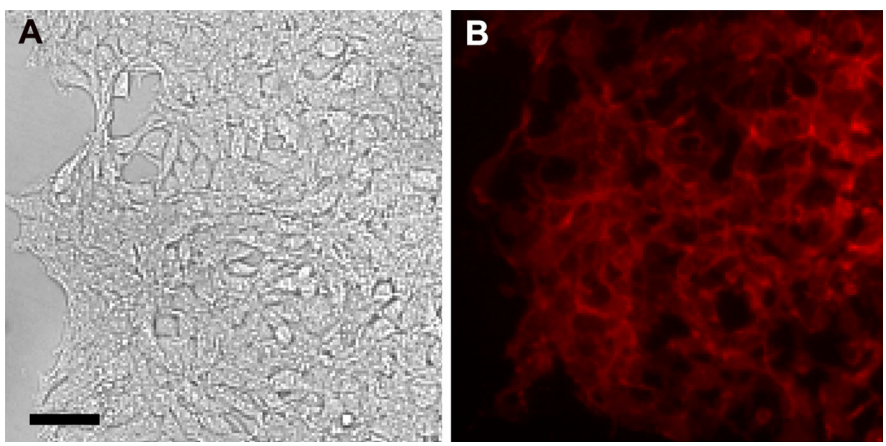

Fig. 1 Co-transfection with Gag–pol and a transfer vector produces stable cell lines. **a** Bright field image of a confluent flask of human embryonic kidney (HEK) 293 cells transfected with SINp-Cherry and GP. **b** Fluorescent cells expressing mCherry ($n = 3$ experiments). Images are in false colour. Scale bars = 40 μm

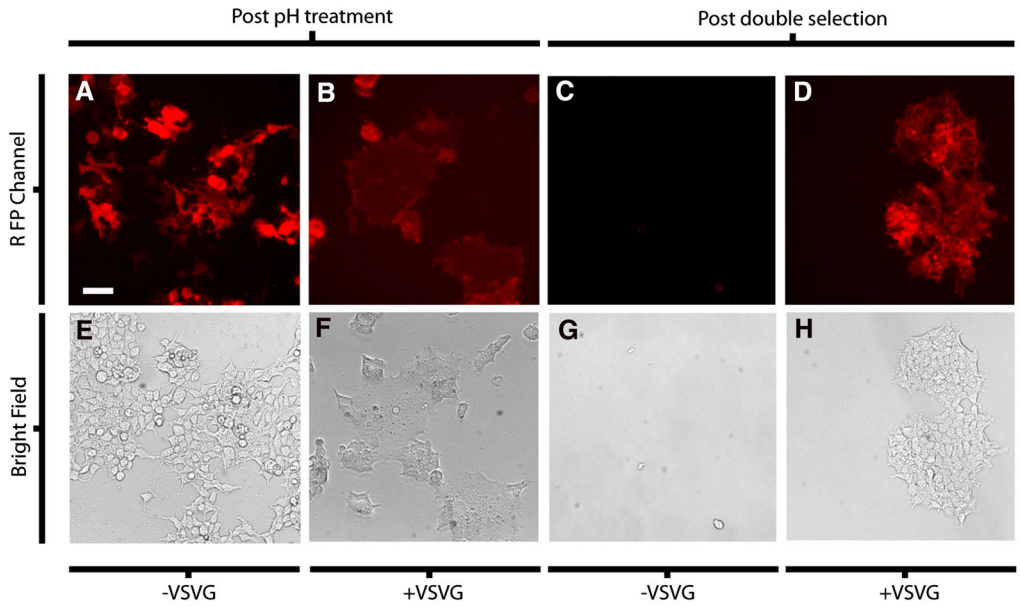

Fig. 2 Lentiviral particles produced by Gag–pol (GP) are not infective in the absence of vesicular stomatitis virus G (VSVG) envelope proteins. Red fluorescent protein (RFP) channel and bright field image after pH treatment for VSVG negative (**a**, **e**) and VSVG positive (**b**, **f**) HEK293 packaging cells respectively. RFP channel and bright field image of HEK293T target cells after co-incubation with VSVG negative (**c**, **g**) and VSVG positive (**d**, **h**) HEK293 packaging cells respectively. Images are in false colour. Scale bar = 40 μm

produced a large fused cell (Fig. 2b, f). HEK293 cells were transfected with SINp-Cherry (containing the blasticidin resistance) and co-incubated with HEK 293T cells (containing neomycin resistance) 24 h post transfection. Once co-incubated, transfected HEK293 packaging cells will export viral particles that will then infect adjacent HEK293 or HEK293T cells. After infection and integration takes place (3 days post co-incubation), selection with both blasticidin and neomycin for 3 weeks would kill every cell except infected target cells (HEK293T cells containing both blasticidin and neomycin resistance). If the viral particles are not infective, the transfer of blasticidin resistance to HEK293T cells will not happen, and double selection would kill all cells. This method of lentiviral infection can infect a target cell using a small-scale culture, but requires a special case in which the target cell contains an antibiotic resistance not found in the packaging cell.

As expected, target HEK293T cells co-incubated with VSVG positive HEK293 packaging cells survived 3 weeks of double selection (Fig. 2d, h), indicating that only lentiviral particles with the VSVG envelope proteins could infect and deliver foreign genetic material. Target cells co-incubated with VSVG negative packaging cells did not survive a 3 weeks selection procedure (Fig. 2c, g). Therefore, as expected in this study and others (Chen et al. 1997; Okimoto et al. 2001), it is highly unlikely that lentiviral particles without envelope proteins are infective within small-scale experiments, and so this possibility cannot account for the initial production of a stable cell line from a co-transfection of GP and SINp-Cherry (Fig. 1a, b).

Inhibiting lentiviral particle budding enhances DNA integration in the packaging cell

To test the possibility of self-infection of the packaging cell, a second GP mutant vector was designed lacking the entire sequence for MA proteins [hereafter GP (NoMA)] (Hearps et al. 2008). Without functional MA proteins, the lentiviral cores produced by the packaging cell cannot bud into an exported viral particle (Yuan et al. 1993) which may further reduce infectivity and increase self-infection. As a control for random integration, a third GP mutant packaging vector was synthesized lacking a functional integrase

gene [hereafter GP (NoIN)], where the 64th aspartic acid residue in the core domain of the protein was substituted for a valine (D64V) (Farazmandfar et al. 2012). The use of this vector would control for the number and concentration of vector used during the co-transfection procedure while impairing integration due to IN. Thus, the only way for integration to occur would be through random integration. Cells were co-transfected with SINp-Cherry and GP or SINp-Cherry and GP (NoMA) or SINp-Cherry and GP (NoIN). The transfected fluorescent cells were collected 24 h post transfection and strained to obtain single isolated cells (Fig. 3b, d, f, h). The isolated cells were allowed to grow into individual colonies, and the number of integrated and non-integrated colonies was counted (illustrated in Supplementary Fig. 3). An integrated colony was identified as a uniformly fluorescent colony, because singly strained fluorescent cells that have integrated the transfer vector into its genome will replicate this gene at every cell division, producing a colony of cells with uniform fluorescence (Fig. 3c, g). In contrast, a non-integrated colony was identified as one containing cells with variable or no fluorescence (Fig. 3e, i) because genes that are not integrated can only be expressed from the transfected plasmid and the concentration of the plasmid approximately halves during every cell division. The ratio of integrated to non-integrated colonies was greatest in the GP (NoMA), then GP, and finally the GP (NoIN) case (24.3 % vs. 14.6 % vs. 0 % respectively, $p < 0.01$, Fig. 3a) ($n = 3$ experiments with over 200 colonies counted).

This result demonstrates three key points. First, random integration leading to the expression of a functional foreign gene is virtually non-existent in small-scale experiments (0 %). Second, the integration seen in the GP case is due to self-infection (14.6 % vs. 0 %) as the effects of random integration and lentiviral particles lacking VSVG are negligible. Third, the enhancement of integration seen in the GP (NoMA) case further supports the possibility that DNA integration can occur via self-infection without viral budding.

Furthermore, to test the applicability of this method in other cell lines, a co-transfection of SINp-Cherry and GP (NoMA) was also performed in Chinese Hamster Ovary (CHO) and Swiss albino mouse embryo (3T3) cells (Supplementary Fig. 4). The

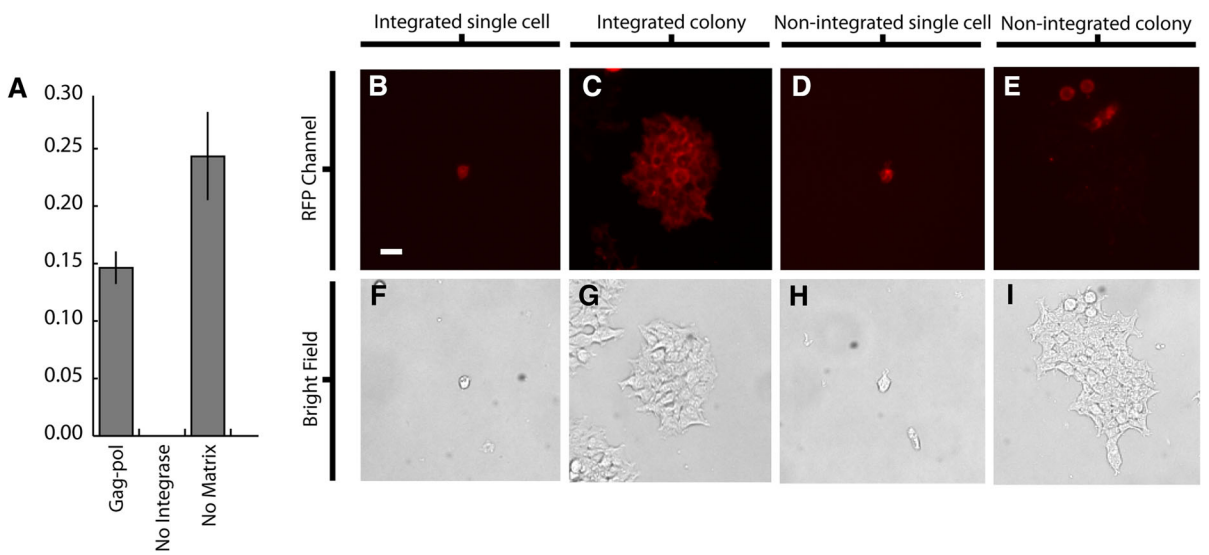

Fig. 3 Inhibiting lentiviral budding enhances DNA integration in the packaging cell. **a** General schematic illustrating the formation of a fluorescent colony by DNA integration and subsequent cell division. **b** *Bar graph* depicting the ratio of integrated to non-integrated colonies counted for each case ($n = 3$, with over 200 colonies counted); *error bars* represent standard deviation. Red fluorescent protein (RFP) channel and bright field image of a singly strained fluorescent cell in the Gag–pol without matrix (GP-NoMA) (**c**, **g**) and negative control (**e**, **i**) case respectively. RFP channel and bright field image of a uniformly fluorescent, integrated colony in the GP-NoMA (**d**, **h**) case, and a non-integrated colony from the negative control case (**f**, **j**) respectively. Images are in false colour. Scale bar = 40 μm

transfection efficiency of these cell lines were lower than HEK293 cells, showing that DNA integration via self-infection is not dependent on the initial efficiency of transfection. With a lower transfection efficiency compared to HEK293 cells, the likelihood of DNA integration via random integration or VSVG-deficient lentivirus would be even lower in CHO and 3T3 cells.

Conclusion

A co-transfection of GP and a transfer vector can produce DNA integration in the packaging cell. It is highly unlikely that DNA integration using this method is due to random integration or infection from VSVG-deficient lentiviral particles. Further, these packaging cells become stably integrated with DNA by a self-infection process that is not dependant on viral budding. If the packaging cell is also the target cell, this method confers two advantages. The first is that genetic integration can be achieved with this method in small-scale experiments, making it more convenient than other transfection or lentiviral infection protocols that require mass cell culturing. The second is that this method does not require the production and handling of any lentiviral particles, and therefore has fewer health and safety considerations.

Supplementary information Supplementary Fig. 1—Vector illustration and mechanism of self infection.

Supplementary Fig. 2—General schematic of the lentiviral infection protocol.

Supplementary Fig. 3—General schematic of fluorescent colony assay.

Supplementary Fig. 4—Co-transfection of SINp-Cherry and GP (NoMA) produces DNA integration.

References

Ambrose Z, Aiken C (2014) HIV-1 uncoating: connection to nuclear entry and regulation by host proteins. Virology 454–455:371–379

Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK (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

Chen ST, Iida A, Guo L, Friedmann T, Yee JK (1996) Generation of packaging cell lines for pseudotyped retroviral vectors of the G protein of vesicular stomatitis virus by using a modified tetracycline inducible system. Proc Natl Acad Sci USA 93:10057–10062

Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ, Marks RM (1997) Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat Med 3:866–871

Clapham PR, McKnight A (2002) Cell surface receptors, virus entry and tropism of primate lentiviruses. J Gen Virol 83:1809–1829

Dalby B, Cates S, Harris A, Ohki EC, Tilkins ML, Price PJ, Ciccarone VC (2004) Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. Methods 33:95–103

Davis L (2012) Basic methods in molecular biology. Elsevier, New York

Farazmandfar T, Khanahmad Shahreza H, Haghshenas MR, Janbabai G, Azadeh H, Mansour Samaei N (2012) Use of integrase-minus lentiviral vector for transient expression. Cell J 14:76–81

Fink D, Wohrer S, Pfeffer M, Tombe T, Ong CJ, Sorensen PH (2010) Ubiquitous expression of the monomeric red fluorescent protein mCherry in transgenic mice. Genesis 48:723–729. doi:10.1002/dvg.20677

Finkelshtein D, Werman A, Novick D, Barak S, Rubinstein M (2013) LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. Proc Natl Acad Sci USA 110:7306–7311

Hearps AC, Wagstaff KM, Piller SC, Jans DA (2008) The *N*-terminal basic domain of the HIV-1 matrix protein does not contain a conventional nuclear localization sequence but is required for DNA binding and protein self-association. Biochemistry 47:2199–2210

Hewinson J, Paton JF, Kasparov S (2013) Viral gene delivery: optimized protocol for production of high titer lentiviral vectors. Methods Mol Biol 998:65–75

Kaplan AH (2002) Assembly of the HIV-1 core particle. AIDS Rev 4:104–111

Merrihew RV, Marburger K, Pennington SL, Roth DB, Wilson JH (1996) High-frequency illegitimate integration of transfected DNA at preintegrated target sites in a mammalian genome. Mol Cell Biol 16:10–18

Mondal Roy S, Sarkar M (2011) Membrane fusion induced by small molecules and ions. J Lipids 2011:528784

Nagaraj S, Mills E, Wong SS, Truong K (2013) Programming membrane fusion and subsequent apoptosis into mammalian cells. ACS Synth Biol 2:173–179. doi:10.1021/sb3000468

Nasri M, Karimi A, Allahbakhshian Farsani M (2014) Production, purification and titration of a lentivirus-based vector for gene delivery purposes. Cytotechnology 66:1031–1038

Okimoto T, Friedmann T, Miyanohara A (2001) VSV-G envelope glycoprotein forms complexes with plasmid DNA and MLV retrovirus-like particles in cell-free conditions and enhances DNA transfection. Mol Ther 4:232–238

Philippe S et al (2006) Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and in vivo. Proc Natl Acad Sci USA 103:17684–17689. doi:10.1073/pnas.0606197103

Quinonez R, Sutton RE (2002) Lentiviral vectors for gene delivery into cells. DNA Cell Biol 21:937–951

Ricks DM, Kutner R, Zhang XY, Welsh DA, Reiser J (2008) Optimized lentiviral transduction of mouse bone marrow-

derived mesenchymal stem cells. Stem Cells Dev 17:441–450

Swift S, Lorens J, Achacoso P, Nolan GP (2001) Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems. Curr Protoc Immunol Chapter 10:Unit 10 17C

Vasquez KM, Marburger K, Intody Z, Wilson JH (2001) Manipulating the mammalian genome by homologous recombination. Proc Natl Acad Sci USA 98:8403–8410

Violin JD, Zhang J, Tsien RY, Newton AC (2003) A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. J Cell Biol 161:899–909

Yu SF et al (1986) Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. Proc Natl Acad Sci USA 83:3194–3198

Yuan X, Yu X, Lee TH, Essex M (1993) Mutations in the *N*-terminal region of human immunodeficiency virus type 1 matrix protein block intracellular transport of the Gag precursor. J Virol 67:6387–6394

Zhang XY, La Russa VF, Reiser J (2004) Transduction of bone-marrow-derived mesenchymal stem cells by using lentivirus vectors pseudotyped with modified RD114 envelope glycoproteins. J Virol 78:1219–1229