International Journal of Pharmaceutical Science Invention
ISSN (Online): 2319 – 6718, ISSN (Print): 2319 – 670X
www.ijpsi.org Volume 6 Issue 6 ǁ June 2017 ǁ PP. 22-28

Comparing Residual Integration Levels of Some Integration-Deficient Lentiviral Vectors

Mohammed Salah Mahmoud Mohammed [1], Ayman Mustafa Abdel-Aziz [2], Eman Mohammed Mohammed Abd-Ella [3]
[1, 2, 3] *(Zoology department, Faculty of Science/ Fayoum University, Egypt)*

Abstract: *Lentiviral vectors (LVs) have many advantageous characteristics making them a good choice in the field of gene therapy. Nevertheless, their integration may lead to detrimental effects. To overcome this problem, lentiviral integration can be targeted through using integration-deficient lentiviral vectors (IDLVs). In this study, an integration-proficient lentiviral vector (IPLV) and a battery of IDLVs with single or multiple mutations affecting integration were produced and their integration levels were compared. eGFP time-course experiment and clonogenic assay were used to make these comparisons. It was found that there was not any significant difference between the residual integration of any of the IDLVs used in this study and that of the standard IDLV; D64V-IDLV. It can be concluded that most IDLV integration is mediated by integrase-independent mechanisms.*

Keywords: *Gene therapy, IDLV, Lentivector, residual integration, triple mutation*

I. Introduction

LVs have many advantageous characteristics making them a good choice in the field of gene therapy, such as comparatively large coding capacity, lower immunogenicity upon in vivo administration compared to other gene delivery vehicles like adenoviral or adeno-associated viral vectors, transduction of non-dividing cells and stable gene expression because of their integration into the host chromosome.

They have been used successfully to deal with many genetic diseases such as thalassemia, chronic lymphocytic leukemia, metachromatic leukodystrophy, and Wiskott-Aldrich syndrome [1-4]. Integrating vectors such as lentivectors have the potential to cause insertional mutagenesis (IM) via inserting their genome into the host genome. For instance, in an ex vivo study using an integrating retroviral vector, it was observed that the transduced bone marrow cells caused leukemia in mice [5]. This result was confirmed by the development of leukemia due to IM of a gammaretroviral vector in the LIM domain only 2 (LMO-2) proto-oncogene [6, 7].

Many enhancements were made to increase the safety of LVs, and one of them was to convert these vectors into IDLVs. IDLVs fail to integrate and instead become episomal circles in the transduced cell, which significantly reduces the risk of causing IM. IDLVs are most commonly generated with mutations in the gene coding for integrase (IN), the viral enzyme encoded by the end sequence of Pol gene, which plays an essential role in integrating viral genome into the host genome.

Viral integration has several steps starting with recognizing conserved attachment sites (att) at both 3' ends of reverse transcribed viral genome and cutting a dinucleotide from both ends (3′-processing). Then, IN bridges the viral cDNA ends within intracellular proteins consisting of viral and cellular proteins. Some cellular proteins contribute in IN activation. The most prominent one is lens epithelium-derived growth factor (LEDGF/p75) [8].

Once in the nucleus, IN activates the strand transfer step in which IN transfers viral DNA to the 5' phosphorylated ends of a double-stranded cut in the target DNA (with 5 nucleotides between cuts of the opposing strands) where it is integrated. After strand transfer step, gaps are then repaired producing 5 base-pair duplications flanking the inserted genome [9, 10] via the action of host cell proteins. There has been a debate about the nature of IDLV integration. In this study, we tried to investigate the nature of residual integration of IDLVs through using different mutation combinations of IN catalytic triad of the catalytic core domain, lens epithelium-derived growth factor (LEDGF/p75) binding domain of IN, and att site.

II. Materials And Methods

2.1. *Plasmids*

pRSV.REV plasmid, pMD2.VSV.G env plasmid and pMDLg_pRRE packaging plasmid possessing wild-type integrase were previously described [11, 12]. pMDLg/pRRE-intD64V_v1 having D64V mutation was prepared via subcloning [13], while pMDLg/pRRE-intD64V_v2 plasmid having the same mutation was prepared by Andrew McNeill and Klaus Wanisch from Yáñez-Muñoz lab (RHUL) via site-directed mutagenesis of pMDLg_pRRE plasmid. The former plasmid carries other mutations outside the catalytic core domain.

R8.91 IN DDE-AAA 2nd generation packaging plasmid carrying (D64A-D164A-E152A) triple mutation in the catalytic domain of IN coding sequence was kindly donated by Dr. Rik Gijsbers (University Leuven, Belgium). pMDLg_pRRE_IN DDE-AAA 3rd generation packaging plasmid was constructed by inserting AgeI-AflII fragment carrying the triple mutation from R8.91 IN DDE-AAA into pMDLg_pRRE plasmid.

Both pMDLg_pRRE and pMDLg_pRRE_IN DDE-AAA packaging plasmids were mutated using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies LDA) as per the manufacturer's protocol to generate Q168A mutation in LEDGF/p75 binding domain of integrase. The resulting plasmids were pMDLg/pRRE_Q168A and pMDLg/pRRE-intDDE-AAA_Q168A, respectively.

Transfer plasmid carrying eGFP gene driven by CMV promoter and puromycin acetyl transferase expressing gene driven by PGK promoter, pRRLsc_C_GFP_P_Puro_W plasmid, was kindly provided by Eric Campeau (Addgene plasmid, 17448) [14]. pRRLsc_C_GFP_P_Puro_W transfer plasmid was also mutated by site-directed mutagenesis to generate two dinucleotide mutations at both 3' att and 5' att sites. The resulting plasmid was pRRLsc_C_GFP_P_Puro_W_m 5P3P. All the plasmids produced in this study were verified by restriction analysis and sequencing (not shown).

2.2. *Bacteria*

Escherichia coli (E. coli) Top 10 strain (Invitrogen) was used for routine plasmid propagation while XL10-Gold ultracompetent strain provided with QuikChange II XL Site-Directed Mutagenesis kit was used for site-directed mutagenesis.

2.3. *Cell growth and maintenance*

Dulbecco's modified Eagle medium (DMEM) (PAA), supplemented with 10% fetal bovine serum (FBS) (Gibco or PAA), 100 units/ml penicillin, and 100 µg/ml streptomycin (PAA), was used for HeLa and HEK-293T cell culture.

2.4. *Lentivector production*

Lentivector production was done using the transient calcium phosphate transfection method [13].

2.5. *Lentivector titration*

After the transduction of HeLa cells with serial dilutions of lentivectors and harvesting the cells after 24 h, genomic DNA extraction was performed using Qiagen DNeasy® tissue kit. Then, Bushman's lentiviral late reverse transcript reaction was used for real-time quantitative polymerase chain reaction (qPCR) as previously mentioned [15].

Functional titer, eGFP titer, was performed by flow cytometry analysis as previously described [13].

2.6. *Measuring eGFP expression*

To measure eGFP expression, HeLa cells (1 x 105 per well in 6-well plate) were transduced with different lentivectors at 1 eGFP multiplicity of infection (MOI). Untransduced cells were also incorporated to be used as a negative control. After 72 h, the cells were harvested and fixed in 1 % paraformaldehyde (PFA) (Sigma-Aldrich) in PBS. Flow cytometer was then used to measure the percent of eGFP positive cells as previously described [13].

To assess eGFP time-course expression, an amount of the harvested cells was fixed and analyzed by flow cytometry and another amount was re-seeded in a fresh medium. This process was repeated at different time points.

2.7. *Clonogenic assay*

After three days from HeLa cell transduction with different lentivectors (eGFP MOI 1), the medium was replaced by puromycin-containing medium (0.4 µg/ml). Then, the medium was changed every two days. After 11 days post-transduction, the colonies were stained with crystal violet and counted blindly.

2.8. *Statistical analyses*

GraphPad Prism software was used to perform statistical analyses. Either One-way or two-way ANOVA analysis was used, followed by Tukey's multiple comparisons test. Data were expressed as mean ± standard deviation and differences were stated to be statistically significant if $P<0.05$. (*) means $P<0.05$, (**) means $P<0.01$, (***) means $P<0.001$, and (****) means $P<0.0001$.

III. Results

3.1. *Lentivector production*

Different lentivectors were produced. Details on these vectors and the plasmids used in their production are shown in **Table 1**.

Table 1: Different lentivectors used in this study and packaging and transfer plasmids involved in their production.

Lentivector name	*Packaging plasmid*	*Transfer plasmid*	*Integration potentiality*
IPLV	pMDLg/pRRE	pRRLsc_C_GFP_P_Puro_W	IPLV
D64V_v1	pMDLg/pRRE-intD64V_v1	pRRLsc_C_GFP_P_Puro_W	IDLV
D64V_v2	pMDLg/pRRE-intD64V_v2	pRRLsc_C_GFP_P_Puro_W	IDLV
DDE_AAA_v1	pR8.91 IN DDE-AAA	pRRLsc_C_GFP_P_Puro_W	IDLV
DDE_AAA_v2	pMDLg/pRRE-intDDE-AAA	pRRLsc_C_GFP_P_Puro_W	IDLV
att--	pMDLg/pRRE	pRRLsc_C_GFP_P_Puro_W_m 5P3P	IDLV
D64V_att--	pMDLg/pRRE-intD64V_v2	pRRLsc_C_GFP_P_Puro_W_m 5P3P	IDLV
Q168A	pMDLg/pRRE_Q168A	pRRLsc_C_GFP_P_Puro_W	IDLV
DDE_AAA_Q168A	pMDLg/pRRE-intDDE-AAA_Q168A	pRRLsc_C_GFP_P_Puro_W	IDLV
Q168A_att--	pMDLg/pRRE_Q168A	pRRLsc_C_GFP_P_Puro_W_m 5P3P	IDLV
DDE-AAA_Q168A_att--	pMDLg/pRRE-intDDE-AAA_Q168A	pRRLsc_C_GFP_P_Puro_W_m 5P3P	IDLV

3.2. *Analysis of the residual integration of IDLVs with catalytic core mutations*

IDLVs with triple mutation (D64A, D116A, and E152A) in the catalytic core domain of IN were compared to IDLVs with D64V catalytic core mutation. IDLV with the D64V mutation had two versions: D64V_v1, which is different from IPLV in having a D64V mutation in the catalytic core domain together with other integrase mutations outside the catalytic core domain, and D64V_v2, which carries a D64V mutation in the catalytic core, as the only difference between this vector and IPLV. IDLV with triple mutation (D64A, D116A, and E152A) had two versions: DDE_AAA v1 produced by using pR8.91 IN DDE-AAA 2nd generation plasmid, and DDE_AAA v2 produced by using pMDLg/pRRE-intDDE-AAA 3rd generation packaging plasmid. Different experiments were performed to compare these vectors.

3.2.1. eGFP time-course experiment of different lentivectors in HeLa cells.

To compare the levels of residual eGFP fluorescence obtained with LVs carrying mutation(s) in the catalytic core domain of IN, a time-course experiment was done by transducing Hela cells with different IDLVs, using eGFP multiplicity of infection (MOI) 1, and measuring eGFP expression at two or three days interval by flow cytometry (**Figure 1**). Phase contrast and fluorescence micrographs were also captured at various time points (**Figure 2**). It was revealed by both flow cytometry and fluorescence microscopy that throughout the experiment, eGFP expression of cells transduced with IPLV, remained within the same range. However, after nine days, eGFP expression in cells transduced with IDLVs was shown to be 0.1-1.0%, which was close to that observed in control cells (0.06%). Moreover, there was not any remarkable difference neither between both versions of D64V-IDLV nor between both versions of DDE-AAA-IDLV. It was also revealed that IDLVs with triple mutation (D64V D116A E152A) did not have a significantly lower residual integration than those with D64V single mutation (D64V).

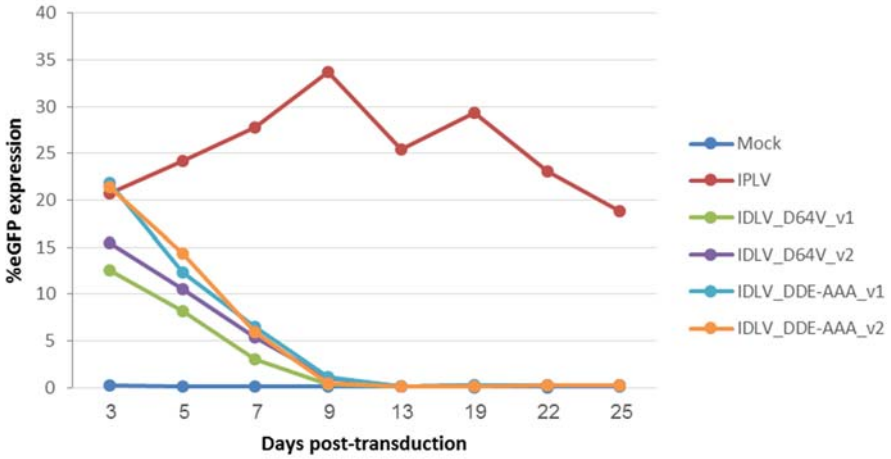

Figure 1: A graph showing the results of the time-course experiment of *eGFP* expression in cells transduced with different lentivectors.Percentages of eGFP-positive cells were determined over time by flow cytometry. Data are represented as (Mean %eGFP-positive cells ± STDEV).

3.2.2. Residual integration of IPLV and IDLVs with catalytic core mutation(s) using clonogenic assay

The clonogenic assay was used as a more sensitive assay to compare residual integration of different LVs. This assay was performed after using IPLV, D64V-v2 IDLV, and DDE-AAA-v2 IDLV in HeLa cells transduction. A preliminary clonogenic assay had not shown any difference between both versions of each IDLV (not shown). The results of this experiment shown in (**Figure 3**) confirmed the results obtained from the eGFP time-course experiment. There was a highly signifJicant difference ($P \leq 0.0001$) between IPLV and any other IDLV with catalytic core mutation(s). On the other hand, there was not any significant difference between D64V-v2 IDLV and DDE-AAA-v2 IDLV.

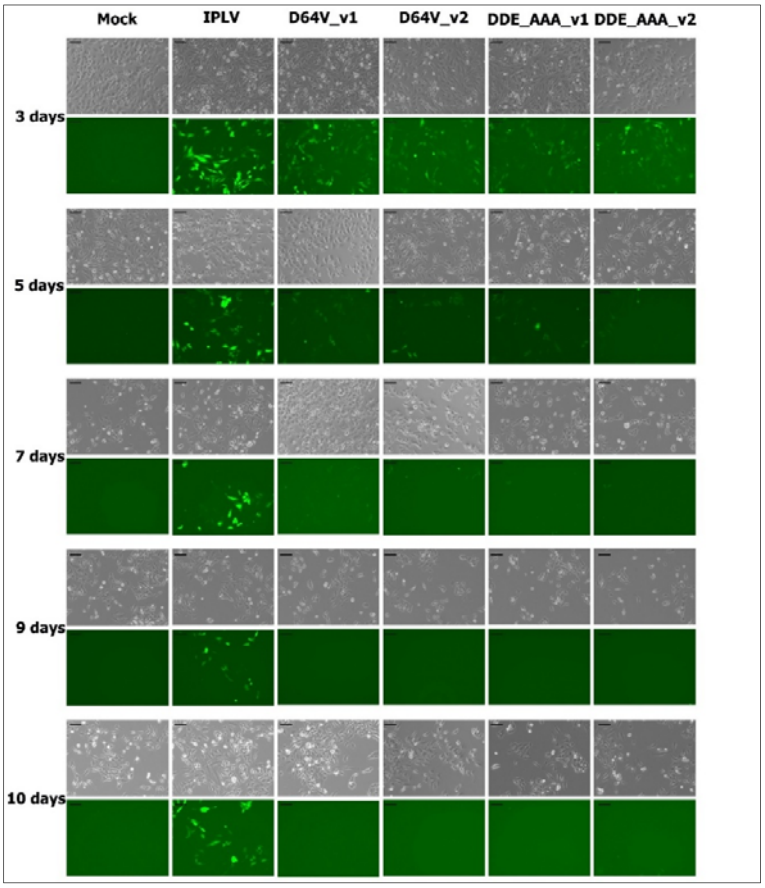

Figure 2: Comparison of eGFP fluorescence in HeLa cells transduced with integrating lentivector or catalytic core mutant IDLVs. Shown are phase contrast and fluorescence photomicrographs of cells transduced with integrating lentivector or catalytic core mutant IDLVs at different time points. Cells transduced with IPLV showed persistent eGFP expression over time in contrary to the remaining IDLVs, which showed the same trend of eGFP expression decrease over time. Scale bar = 100 µm.

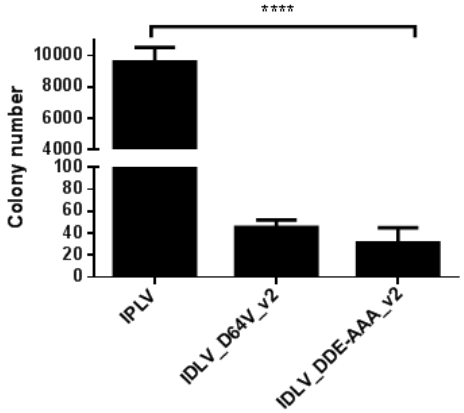

Figure 3: Puromycin-resistant colony formation after transduction using IPLV and IDLVs with catalytic core mutation(s) at eGFP MOI 1. Data are represented as (Mean colony number ± STDEV); Analysis was done using one-way ANOVA followed by Tukey's multiple comparisons test. **** indicates ($P \leq 0.0001$).

3.3. *Clonogenic assay after using IPLV and IDLVs with different mutation combinations of catalytic core mutation(s), att sites mutations and LEDGF/p75 binding domain mutation.*

Novel IDLVs with different mutation combinations of catalytic core mutation(s), att sites mutations and LEDGF/p75 binding domain mutation were also compared with the standard D64V IDLV. LVs tested in this experiment included: IPLV; D64V_v2, IDLV with D64V mutation; Q168A, IDLV with Q168A mutation in LEDGF/p75 binding domain; DDE_AAA_Q168A, IDLV with D64A_D116A_E152A triple mutation and Q168A mutation in LEDGF/p75 binding domain; att--, IDLV with dinucleotide mutation at both attachment sites; D64V_att--, IDLV with D64V mutation and dinucleotide mutation at both attachment sites; DDE-AAA_att--, IDLV with D64A_D116A_E152A triple mutation and dinucleotide mutation at both attachment sites; Q168A_att--, IDLV with Q168A in LEDGF/p75 binding domain plus dinucleotide mutation at both attachment sites; DDE_AAA_Q168A_att--, IDLV with dinucleotide mutation at both attachment sites, D64A_D116A_E152A triple mutation and Q168A mutation in LEDGF/p75 binding domain.

The results of this experiment represented in (**Figure 4**) can be summarized in the following points:

- All IDLVs had colony counts significantly lower than that of the IPLV ($P \leq 0.01$ for Q168A-IDLV and $P \leq 0.001$ for the remaining IDLVs).
- Q168A-IDLV had a non-significantly higher residual integration than any other IDLV.

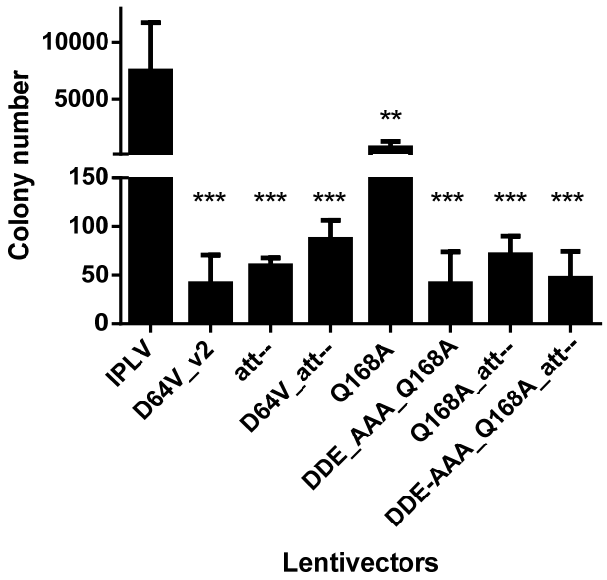

Figure 4: Puromycin-resistant colony formation after transduction using IPLV and IDLVs with different mutation combinations of catalytic core mutation(s), *att* sites mutations and LEDGF/p75 binding domain mutation at MOI 1. Data are represented as (Mean colony number ± STDEV); Analysis was done using one-way ANOVA followed by Tukey's multiple comparisons test. ** indicates ($P \leq 0.01$) and *** indicates $P \leq 0.001$.

IV. Discussion

In this study, IN mutants with different alterations of catalytic site residues were compared. Moreover, IDLVs having combinations of mutations such as Q168A mutation in LEDGF/p75 binding domain of IN and mutations at both sequences coding for the attachment sites (att sites) were also assessed. These LVs were compared with the standard D64V-IDLV (D64V-v2) and with LVs carrying one type of mutations to assess if there was a synergistic effect of combining different mutations.

By using flow cytometry as an initial approach, IPLV eGFP gene expression remained within the same range throughout the experiment in agreement with previous studies [13, 16, 17] as gene cassette is integrated and hence expressed along with host genome in the transduced cells and their progenies. This result was supported by phase contrast and fluorescence micrographs. However, the eGFP fluorescence of HeLa cells transduced with different IDLVs was shown to be 0.1-1.0% without any significant difference between them.

The clonogenic assay was then used as a more sensitive tool to compare residual integration levels. Integration levels of D64V IDLV and DDE_AAA IDLV were 0.47 and 0.33%, respectively compared to IPLV. This indicated that an IDLV with a triple mutation at the catalytic triad is not better than IDLV with a single point mutation at the catalytic triad in terms of residual integration. Previous studies concerning D64V IDLV showed reductions of 3-4 logs in the number of resistant colonies [18].

IDLV possessing att site mutations showed persistent transfer gene expression in non-dividing cells and transient transfer gene expression in dividing cells [19]. In our study, att site mutations led to a similar reduction in integration frequency as IN catalytic active site mutations. According to clonogenic assay, residual integration of att-- IDLV was found to be 0.8% compared to IPLV integration. Previous studies showed lower residual integration compared to IPLV by about 100 fold [20] or 200 fold [19]. We have confirmed that LVs with mutations at both the integrase catalytic site and attachment sites had no synergistic effect on integration efficiency, in agreement with previous studies [19, 21, 22].

The interaction between LEDGF/p75 and IN can be targeted in gene therapy field to decrease integration as well as in the field of antiretroviral drug development. It has been shown in a previous in vitro study [23] that Q168A integrase mutation in LEDGF/p75 binding domain affected IN-LEDGF/p75 interaction and hence prevented tethering between integrase and chromosomes and subsequently lowered the integration to a level, which was quite similar to that of D116A integrase mutation. However, in our study, we found that Q168A-IDLV had 9.5% residual integration compared to IPLV according to clonogenic assay, which was more than that of any other IDLV in this study (0.5% -1.2% residual integration). This result indicated that the role of LEDGFP/p75 in integration is accessory in contrast to the roles of both the conserved catalytic triad (D64, D116, and E152) and the conserved sequences at both att sites, which are crucial for integration.

All IDLVs used in this study were shown to have significantly lower integration levels compared to IPLV ($P \leq 0.0001$) with slight differences between them. This means that these IDLVs, excluding Q168A-IDLV, are good candidates for further research to assess the safety and integration site preference of each of them. No synergy effect was found between different mutations. The only exception was Q168A, which gave lower residual integration when combined with other mutation(s) in IDLV production. This supports the conclusion that the residual integration of proficient IDLVs is IN-independent. DNA repair mechanisms naturally exist to repair double-strand breaks (DSBs) were proposed to be the cause of illegitimate integration of IDLV [24]. This was supported by the fact that DSBs usually uptake extrachromosomal DNA fragments, and finding that an IDLV was preferably integrated into DSBs pre-existing in human osteosarcoma U2OS cells [25].

V. Conclusion

This study gives a better understanding of the residual integration of LVs. The residual integration of IDLV mutants used in this study appeared to be integrase-independent, as it could not be reduced by combining mutations. The only exception was the Q168A IDLV. This exception confirms that integrase with Q168A mutation in the binding site for LEDGF/p7 is partially functional. More studies are needed to compare the sites of integration of different IDLVs.

Acknowledgements

This work was funded by the Egyptian government, missions sector. We would like to thank Dr. Rik Gijsbers (University Leuven, Belgium) for kindly providing us with R8.91 IN DDE-AAA 2nd generation packaging plasmid carrying integrase triple mutation (D64A, D116A, and E152A).

References

[1] Cavazzana-Calvo, M., et al., *Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia.* Nature, 2010. **467**(7313): p. 318-22.
[2] Porter, D.L., et al., *Chimeric antigen receptor–modified T cells in chronic lymphoid leukemia.* New England Journal of Medicine, 2011. **365**(8): p. 725-733.
[3] Aiuti, A., et al., *Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome.* Science, 2013. **341**(6148): p. 1233151.
[4] Biffi, A., et al., *Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy.* Science, 2013. **341**(6148): p. 1233158.
[5] Li, Z., et al., *Murine leukemia induced by retroviral gene marking.* Science, 2002. **296**(5567): p. 497.
[6] Hacein-Bey-Abina, S., et al., *LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1.* Science, 2003. **302**(5644): p. 415-419.
[7] Howe, S.J., et al., *Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients.* The Journal of clinical investigation, 2008. **118**(9): p. 3143-3150.
[8] Cherepanov, P., et al., *HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells.* Journal of Biological Chemistry, 2003. **278**(1): p. 372-381.
[9] Katz, R.A., et al., *The avian retroviral IN protein is both necessary and sufficient for integrative recombination in vitro.* Cell, 1990. **63**(1): p. 87-95.
[10] Mizuuchi, K., *Polynucleotidyl transfer reactions in transpositional DNA recombination.* Journal of Biological Chemistry, 1992. **267**: p. 21273-21273.
[11] Naldini, L., et al., *In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector.* Science, 1996. **272**(5259): p. 263-267.
[12] Dull, T., et al., *A third-generation lentivirus vector with a conditional packaging system.* Journal of virology, 1998. **72**(11): p. 8463-8471.
[13] Yáñez-Muñoz, R.J., et al., *Effective gene therapy with nonintegrating lentiviral vectors.* Nature medicine, 2006. **12**(3): p. 348-353.

[14] Campeau, E., et al., *A versatile viral system for expression and depletion of proteins in mammalian cells.* PLoS One, 2009. **4**(8): p. e6529.
[15] Miller, M.D., C.M. Farnet, and F.D. Bushman, *Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition.* J Virol, 1997. **71**(7): p. 5382-90.
[16] Yang, G., et al., *Integration-deficient lentivectors: an effective strategy to purify and differentiate human embryonic stem cell-derived hepatic progenitors.* BMC Biology, 2013. **11**(1): p. 86.
[17] Wanisch, K. and R.J. Yáñez-Muñoz, *Integration-deficient lentiviral vectors: a slow coming of age.* Molecular Therapy, 2009. **17**(8): p. 1316-1332.
[18] Leavitt, A., et al., *Human immunodeficiency virus type 1 integrase mutants retain in vitro integrase activity yet fail to integrate viral DNA efficiently during infection.* J Virol, 1996. **70**: p. 721 - 728.
[19] Apolonia, L., et al., *Stable gene transfer to muscle using non-integrating lentiviral vectors.* Molecular Therapy, 2007. **15**(11): p. 1947-1954.
[20] Masuda, T., M.J. Kuroda, and S. Harada, *Specific and Independent Recognition of U3 and U5att Sites by Human Immunodeficiency Virus Type 1 Integrase In Vivo.* Journal of virology, 1998. **72**(10): p. 8396-8402.
[21] Nightingale, S.J., et al., *Transient gene expression by nonintegrating lentiviral vectors.* Molecular Therapy, 2006. **13**(6): p. 1121-1132.
[22] Philippe, S., et al., *Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and in vivo.* Proc Natl Acad Sci U S A, 2006. **103**(47): p. 17684-9.
[23] Emiliani, S., et al., *Integrase mutants defective for interaction with LEDGF/p75 are impaired in chromosome tethering and HIV-1 replication.* Journal of Biological Chemistry, 2005. **280**(27): p. 25517-25523.
[24] Gaur, M. and A. Leavitt, *Mutations in the human immunodeficiency virus type 1 integrase D, D(35)E motif do not eliminate provirus formation.* J Virol, 1998. **72**: p. 4678 - 4685.
[25] Cornu, T.I. and T. Cathomen, *Targeted Genome Modifications Using Integrase‐ deficient Lentiviral Vectors.* Molecular Therapy, 2007. **15**(12): p. 2107-2113.