Combinatorial RNAi against HIV-1

Liu, Y.P.

Chapter 7

Lentiviral vector delivery of RNAi inducers against HIV-1

Ying Poi Liu
Ben Berkhout

Laboratory of Experimental Virology,
Department of Medical Microbiology,
Center for Infection and Immunity Amsterdam (CINIMA),
Academic Medical Center, University of Amsterdam,
The Netherlands

Adapted from: Lentiviral delivery of RNAi effectors against HIV-1, Current Topics in Medicinal Chemistry (2009) in press

This chapter covers the recent progress in RNAi-based approaches against HIV-1 using lentiviral vectors as a delivery system. The potential for a clinical gene therapy application will be discussed.

Lentiviral vectors

For gene delivery, recombinant viral vectors based on viruses are very attractive because of their broad cell tropism and efficiency in delivering the vector genome and its therapeutic cargo. Lentiviral vectors that are based on the HIV-1 genome are attractive delivery systems for stable expression of RNAi inducers due to their ability to transduce many cell types, including hematopoietic stem cells and nondividing cells (1,2). In addition, the lentiviral vector becomes stably integrated into the host cell genome, thereby allowing stable long-term transgene expression (3). Furthermore, lentiviral vectors tend to integrate within introns of active transcriptional units, thereby limiting their potential to cause insertional oncogenesis (4). The lower risk of insertional oncogenesis compared to retroviral vectors is also reflected by the fact that the development of HIV-1 induced leukemia is never seen in infected patients (5,6).

Lentiviral vectors were originally designed as replication-defective, hybrid viral particles consisting of the core proteins and enzymes of HIV-1 and the Envelope protein of a different virus, most often the glycoprotein of vesicular stomatitis virus (VSV) due to its broad target cell tropism (Fig. 1) (7-9). The packaging functions are provided by an expression plasmid that encodes the Gag and Pol proteins. Rev protein is made from a separate plasmid to allow cytoplasmic expression of the vector genome. A separate construct expresses the Envelope protein that is incorporated into the vector particles (pseudotyping) and that allows viral entry into a wide spectrum of target cells (10). The biosafety is improved by providing separate packaging plasmids during lentiviral vector production because this will reduce the probability of the generation of a replication-competent virus through recombination (11). Further improvement of the biosafety was achieved by construction of self-inactivating (SIN) vectors (12,13). These vectors contain a deletion in the U3 region of the 3′ long-terminal repeat (LTR), which is transferred to the 5′ LTR promoter during reverse transcription (Fig. 1) (14). As this deletion removes the viral enhancer and promoter sequences, this modification leads to transcriptional inactivation of the LTR promoter in transduced cells. This self-inactivating vector design diminishes the risk of oncogene activation by promoter insertion and substantially reduces the risk of vector mobilization and recombination with wild-type virus (15).

Another biosafety related issue is the administration of a minimal amount of viral particles to prevent cytotoxicity due to e.g. inflammation, tissue damage and other severe side effects. Therefore viral particles were developed that have optimized transduction efficiency, such that a minimal dose of viral particles is sufficient to obtain a therapeutic effect. A central polypurine tract

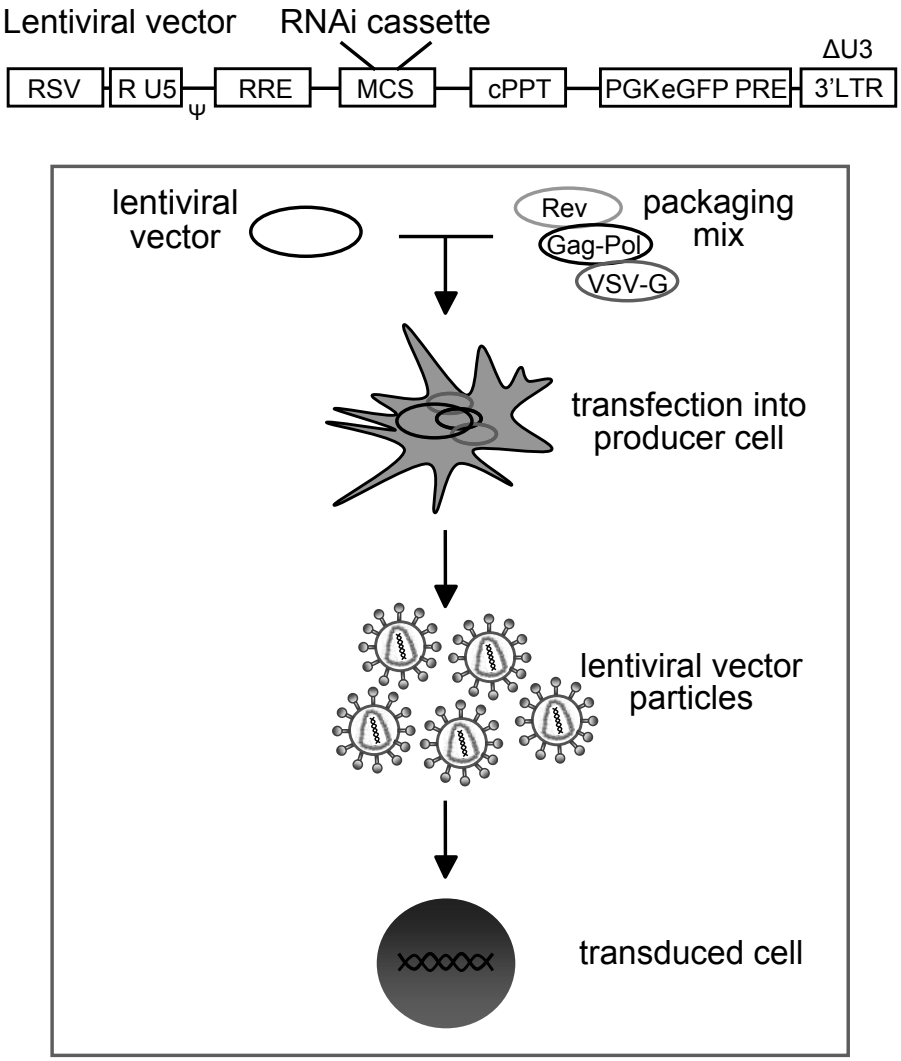

Figure 1. Self-inactivating lentiviral vectors for stable gene delivery. The lentiviral vector design is shown on top. MCS indicates the multiple cloning site in which the RNAi cassette is cloned. The lentiviral vector production and transduction procedure is indicated below. See text for further details.

(cPPT) sequence and a posttranscriptional regulatory element (PRE) from the genome of the woodchuck hepatitis virus have been introduced into the vector backbone to enhance the transduction efficiency (Fig. 1). Insertion of the cPPT, which is a sequence embedded within the pol gene of HIV-1, results in enhanced nuclear translocation of the vector genome, thus increasing the transduction efficiency for a range of primary human cell types (16,17), including peripheral blood lymphocytes, macrophages, umbilical cord endothelial cells,

hematopoietic stem cells and T lymphocytes (18-20). Incorporation of the PRE sequence near the 3′ end of the vector enhances transgene expression by increasing both nuclear and cytoplasmic mRNA levels early during the biogenesis of RNA transcripts (21-25).

For lentiviral vector production, producer cells such as the SV40 large T antigen expressing HEK293T cells are transfected with 4 different plasmids: the lentiviral construct expressing the RNAi-inducing transgene and plasmids expressing Gag-Pol, Rev and VSV-G protein (Fig. 1). The replication-incompetent vector particles can perform a single round infection and deliver the therapeutic cargo in the chromosome of the target cells (transduction).

Gene therapy for HIV-1 using lentiviral vectors

To date, lentiviral vectors have been shown to be effective for *in vivo* delivery, integration and stable expression of transgenes in the central nervous system, the hematopoietic system, hepatocytes and myocytes (7,26-30). The fact that lentiviral vectors can efficiently transduce the hematopoietic system makes this delivery system very suitable for a gene therapy approach against HIV-1. An *ex vivo* gene therapy procedure to deliver antiviral genes to HIV-1 susceptible cells is depicted in Fig. 2. Hematopoietic CD34+ progenitor cells (after G-CSF mobilization) or CD4+ T cells will be isolated from peripheral blood after apheresis. These can be either autologous or from a donor, e.g. an identical twin. The cells will be transduced with lentiviral vectors expressing the RNAi effectors against HIV-1. Subsequently, the transduced cells are re-infused into the patient, where they will resist HIV-1 infection and hopefully prevent the gradual collapse of the immune system. As CD4+ T cells have a limited life span periodic infusions will be required. Therefore, it is perhaps more desirable to transduce CD34+ hematopoietic stem cells because these cells will persist to give rise to all myeloid and lymphoid lineages, and thus this approach ideally only needs a single infusion. Engraftment of autologous transduced CD34+ cells will not only result in a steady production of HIV-1 resistant T cells, but also other HIV-1 susceptible cells, e.g. monocytes and macrophages.

Some years ago, the development of T cell leukemia in a hematopoietic stem cell gene therapy trial for X-linked inherited immunodeficiency made researchers aware of the possible risks of a gene therapy with integrating retroviral vectors (31). Notably, the majority of the individuals subjected to the treatment were successfully cured by a single gene therapy from a deadly disease. The leukemia cases were due to integration of the gamma retroviral vectors in close proximity to the LMO2 proto-oncogene. This resulted in increased expression of this proto-oncogene and subsequently oncogenic transformation of a subset of T cells that expanded to cause leukemia in these patients (32).

Although previous results were disappointing, currently two phase 1 clinical trials using retroviral vectors have demonstrated that *ex vivo* transduction of

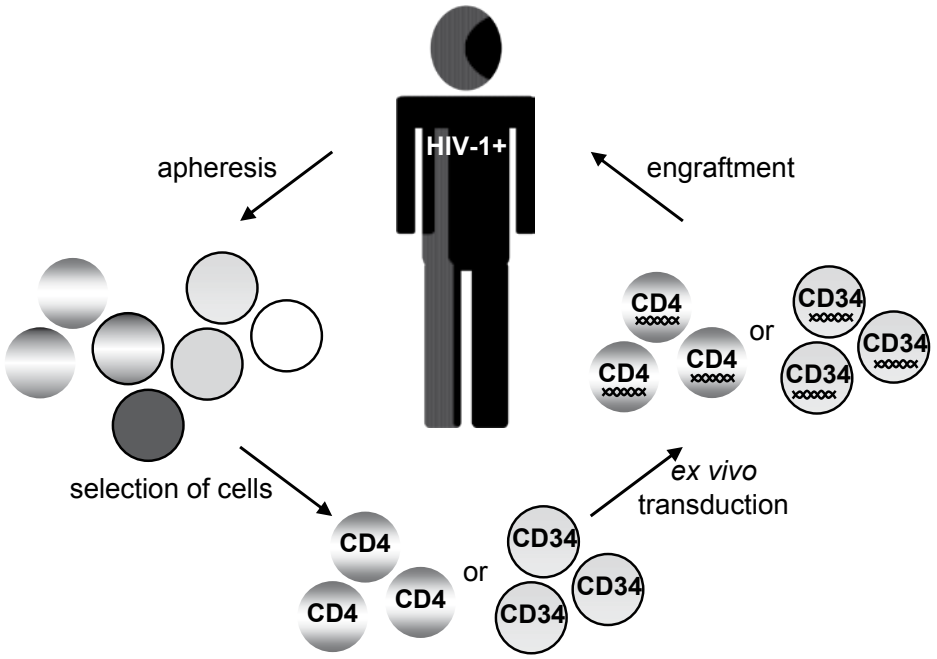

Figure 2. ***Ex vivo*** **gene therapy for HIV-1 infected patients.** See text for details.

either CD4+ T cells (33) or CD34+ hematopoietic stem cells (34) and reinfusion of the transduced cells is feasible and safe. Recently, a phase 2 gene therapy trial based on retroviral vectors encoding anti-HIV ribozymes demonstrated that gene therapy is safe in HIV-1 infected individuals (35). A clinical trial was conducted in 2003 using lentiviral vectors encoding an antisense transcript against HIV-1 (36). In this study, the CD4+ T cells were treated with the lentiviral vector *ex vivo* and after expansion reinfused into the patients. In this study, no adverse events were observed, which led to a subsequent phase I/II trial involving multiple infusions of transduced T lymphocytes in early-stage HIV-1 infected individuals with well-controlled viral loads. Studies were conducted to determine the integration sites of these vectors in CD4+ T cells for three patients before and after infusion of the transduced cells (37). The results obtained correlated well with the known HIV-1 integration pattern. This finding is highly encouraging for a gene therapy approach using lentiviral vectors, and this idea is further supported by the fact that T cell malignancies due to HIV-1 integration have never been observed in infected patients.

Recently, a phase I clinical trial has been initiated with lentiviral vectors expressing a shRNA targeting HIV tat and rev, a nucleolar-localizing TAR decoy and an anti-CCR5 ribozyme (38). The transduced stem cells have been infused into HIV-positive patients in a trial that uses autologous bone marrow transplantation to treat AIDS-related lymphoma.

Lentiviral vectors expressing RNAi inducers against HIV-1

For the development of a clinical application of gene therapy it is essential that the vector can be produced to high titers and that the vector is genetically stable. Lentiviral vectors are efficient in transducing CD4+ T cells and CD34+ blood stem cells. However, the lentiviral vector system is based on HIV-1, which may complicate its use as a vehicle to deliver anti-HIV-1 RNAi inducers. General lentivirus properties may also complicate things. For instance, HIV-1 is well-known to be recombination-prone. This characteristic led to recombination within the lentiviral vector when multiple shRNA expression cassettes with repeated promoter sequences were used (39). Such recombination-mediated deletion of vector sequences could be avoided by expression of the shRNAs from different promoters, which improved the vector genome stability (40).

A specific problem may arise when the anti-HIV-1 siRNAs target sequences of the packaging plasmids or the lentiviral vector genome. It has been reported that targeting of the Gag-Pol and Rev mRNAs of the packaging system can reduce the titer of the lentiviral vector (41,42). Previously, we addressed this issue by determining the capsid and transduction titers of a set of anti-HIV-1 shRNAs (43). The titer of lentiviral vectors encoding shRNAs against the Rev mRNA was not reduced, which is likely due to the presence of an excess Rev protein. A severe reduction in vector titers was measured due to Gag-Pol targeting by some of the antiviral shRNAs. As the RNAi mechanism is sequence-specific, the usage of a modified Gag-Pol construct with multiple silent codon changes will solve this problem (44).

A dramatic reduction in vector titers has also been observed when shRNAs are targeting sequences in the lentiviral vector genome (43) (chapter 6). This problem can be avoided by careful selection of shRNAs that do not target parts of the lentiviral vector backbone. When the vector RNA genome is targeted, a dramatic reduction of the transduction titer will be measured, although the actual amount of produced vector particles (production titer) will not be affected. We tested several strategies to abrogate the RNAi response against the vector genome in the producer cell by co-transfection of a variety of reagents (chapter 6). We demonstrated that the vector titers could be repaired with an excess shRNAs, siRNAs against Dicer and shRNA against Drosha.

Since the lentiviral vector has an RNA genome, any shRNA or miRNA that is also part of the lentiviral vector can potentially target this vector sequence. We previously did not observe a reduction in titer due to such self-targeting (43). This is likely due to the presence of a perfect hairpin structure (shRNA) that reduces the target accessibility for RISC and thereby prevents RNAi attack (45-48). Strikingly, Poluri et al. did report that lentiviral vectors encoding shRNAs have reduced titers due to self-targeting (49). Inhibition of the RNAi pathway by overexpression of Nodamura-virus B2 protein or Adenovirus VA.1 RNA as RNAi suppressors alleviated the titer reduction. It is currently unclear what determines the different outcomes of these studies.

For miRNA-expressing lentiviral vectors, the situation is slightly more complex. First, a reduction in titer could be caused by Drosha processing of the miRNA as part of the vector RNA genome. Indeed, we showed that knocking down Drosha resulted in titer improvement of this type of vector (chapter 6). However, the titer repair was only partial, indicating that there is at least one other problem. Preliminary results indicate that the design of the miRNA cassette (orientation, type of promoter etc.) is of critical importance for efficient use of the lentiviral vector system.

Future perspectives of RNAi therapeutics delivered by lentiviral vectors

To apply RNAi in the clinic, critical issues including efficacy and safety have to be carefully examined. Safety is the major concern, since RNAi inducers can cause undesired side-effects. Induction of IFN has been associated with the expression of dsRNA larger than 30 bp. Recently, it has been shown that shRNAs and siRNAs can also evoke this reaction, which is dose- and sequence-dependent (50-52). Interestingly, a recent report showed that miRNA-like expression systems successfully avoid induction of the interferon-response gene Oas1, which otherwise occurs when shRNA constructs are used (53).

Another problem is the induction of off-target effects in which siRNAs silence partially complementary transcripts through a miRNA-like translational repression mechanism (54). Such an effect requires pairing of the seed area of the siRNA/miRNA guide strand with small complementary stretches in the 3′ UTR of non-target mRNAs. Thus, in a combinatorial RNAi approach, the chance of potential off-target effects will increase, which could affect cell physiology.

Recently, massive expression of shRNAs has been shown to cause fatality in mice through saturation of the endogenous miRNA pathway (55). To minimize the risk of toxicity, the concentration of RNAi inducer should ideally be fine-tuned. This can be done with a cell-type specific polymerase II promoter, which has several advantages. First, expression of the RNAi inducer will only occur in the cells of interest. Second, this type of promoter will allow regulated shRNA/miRNA expression. The use of miRNA-like hairpins has been shown to enhance the RNAi efficacy and safety compared to an shRNA, thus allowing a lower dose of RNAi inducer (56,57). In a combinatorial RNAi setting, using the lowest efficacious dose is crucial to reduce the risk of cytotoxicity. Thus, the use of miRNA-like hairpins that are expressed from inducible or tissue-specific promoters may be a suitable approach to enhance RNAi activity and at the same time reduce toxicity. Future studies are required to test whether the expression of multiple miRNAs will cause similar unwanted side-effects. This should also be tested in appropriate *in vivo* animal models.

Another genuine concern is the possibility that the lentiviral vector will integrate near an oncogene, which may result in increased oncogene expression. However, the currently used self-inactivating lentiviral vectors lack pro-

moter/enhancer motifs and thus significantly reduce this risk. It is currently unknown whether other promoters introduced into the lentiviral vectors can exhibit long-range enhancer effects or drive read-through transcription into adjacent chromosome sequences of the host. The recent observation that lentiviral vector integration sites in CD4+ T cells are very similar to those in HIV-1 infected cells is encouraging since HIV-1 infection has not been associated with integration-induced T cell malignancies. Currently, several clinical trials are ongoing using lentiviral vectors and hematopoietic stem cells. It remains important to test whether the lentiviral vector integration sites are similar in these cells compared to CD4+ T cells. More careful analyses should be performed to address this issue.

Initial results suggest that lentiviral vector delivery of RNAi inducers to CD4+ T cells is effective and safe (36). Further studies must be carried out to better understand the side effects, efficacy and safety of RNAi therapies against HIV-1 *in vivo*. Given the high interest in this field, more clinical applications for RNAi-based treatments will likely emerge in the coming years.

References

1. Gervaix,A., West,D., Leoni,L.M., Richman,D.D., Wong-Staal,F. and Corbeil,J. (1997) A new reporter cell line to monitor HIV infection and drug susceptibility *in vitro*. *Proc Natl Acad Sci U S A*, **94**, 4653-4658.
2. Yam,P.Y., Li,S., Wu,J., Hu,J., Zaia,J.A. and Yee,J.K. (2002) Design of HIV vectors for efficient gene delivery into human hematopoietic cells. *Mol Ther.*, **5**, 479-484.
3. Greber,U.F. and Fassati,A. (2003) Nuclear import of viral DNA genomes. *Traffic.*, **4**, 136-143.
4. Wu,X., Li,Y., Crise,B. and Burgess,S.M. (2003) Transcription start regions in the human genome are favored targets for MLV integration. *Science*, **300**, 1749-1751.
5. Chun,T.W., Carruth,L., Finzi,D., Shen,X., DiGiuseppe,J.A., Taylor,H., Hermankova,M., Chadwick,K., Margolick,J., Quinn,T.C. *et al.* (1997) Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature*, **387**, 183-188.
6. Rouzine,I.M. and Coffin,J.M. (1999) Search for the mechanism of genetic variation in the pro gene of human immunodeficiency virus. *J Virol*, **73**, 8167-8178.
7. Naldini,L., Blomer,U., Gallay,P., Ory,D., Mulligan,R., Gage,F.H., Verma,I.M. and Trono,D. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*, **272**, 263-267.
8. 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.
9. 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.U.S.A*, **93**, 15266-15271.
10. Naldini,L. (1998) Lentiviruses as gene transfer agents for delivery to non-dividing cells. *Curr. Opin.Biotechnol.*, **9**, 457-463.
11. Dull,T., Zufferey,R., Kelly,M., Mandel,R.J., Nguyen,M., Trono,D. and Naldini,L. (1998) A third-generation lentivirus vector with a conditional packaging system. *J Virol*, **72**, 8463-8471.
12. 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.

13. Zufferey,R., Dull,T., Mandel,R.J., Bukovsky,A., Quiroz,D., Naldini,L. and Trono,D. (1998) Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. *J Virol*, **72**, 9873-9880.
14. Iwakuma,T., Cui,Y. and Chang,L.-J. (1999) Self-inactivating lentiviral vectors with U3 and U5 modifications. *Virol.*, **261**, 120-132.
15. Bukovsky,A.A., Song,J.P. and Naldini,L. (1999) Interaction of human immunodeficiency virus-derived vectors with wild- type virus in transduced cells. *J.Virol.*, **73**, 7087-7092.
16. Charneau,P., Mirambeau,G., Roux,P., Paulous,S., Buc,H. and Clavel,F. (1994) HIV-1 reverse transcription. A termination step at the center of the genome. *J Mol Biol*, **241**, 651-662.
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. 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.
19. Sirven,A., Pflumio,F., Zennou,V., Titeux,M., Vainchenker,W., Coulombel,L., Dubart-Kupperschmitt,A. and Charneau,P. (2000) The human immunodeficiency virus type-1 central DNA flap is a crucial determinant for lentiviral vector nuclear import and gene transduction of human hematopoietic stem cells. *Blood*, **96**, 4103-4110.
20. Dardalhon,V., Herpers,B., Noraz,N., Pflumio,F., Guetard,D., Leveau,C., Dubart-Kupperschmitt,A., Charneau,P. and Taylor,N. (2001) Lentivirus-mediated gene transfer in primary T cells is enhanced by a central DNA flap. *Gene Ther.*, **8**, 190-198.
21. Patzel,V. and Sczakiel,G. (1997) The hepatitis B virus posttranscriptional regulatory element contains a highly stable RNA secondary structure. *Biochem.Biophys.Res.Commun.*, **231**, 864-867.
22. Donello,J.E., Loeb,J.E. and Hope,T.J. (1998) Woodchuck hepatitis virus contains a tripartite post-transcriptional regulatory element. *J.Virol.*, **72**, 5085-5092.
23. Deglon,N., Tseng,J.L., Bensadoun,J.C., Zurn,A.D., Arsenijevic,Y., Pereira de,A.L., Zufferey,R., Trono,D. and Aebischer,P. (2000) Self-inactivating lentiviral vectors with enhanced transgene expression as potential gene transfer system in Parkinson's disease. *Hum.Gene Ther.*, **11**, 179-190.
24. 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.
25. Higashimoto,T., Urbinati,F., Perumbeti,A., Jiang,G., Zarzuela,A., Chang,L.J., Kohn,D.B. and Malik,P. (2007) The woodchuck hepatitis virus post-transcriptional regulatory element reduces readthrough transcription from retroviral vectors. *Gene Ther.*, **14**, 1298-1304.
26. Follenzi,A., Battaglia,M., Lombardo,A., Annoni,A., Roncarolo,M.G. and Naldini,L. (2004) Targeting lentiviral vector expression to hepatocytes limits transgene-specific immune response and establishes long-term expression of human antihemophilic factor IX in mice. *Blood*, **103**, 3700-3709.
27. Kordower,J.H., Emborg,M.E., Bloch,J., Ma,S.Y., Chu,Y., Leventhal,L., McBride,J., Chen,E.Y., Palfi,S., Roitberg,B.Z. *et al.* (2000) Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science*, **290**, 767-773.
28. 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.
29. Nguyen,T.H., Oberholzer,J., Birraux,J., Majno,P., Morel,P. and Trono,D. (2002) Highly efficient lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes. *Mol.Ther.*, **6**, 199-209.
30. 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.
31. Gaspar,H.B. and Thrasher,A.J. (2005) Gene therapy for severe combined immunodeficiencies. *Expert.Opin.Biol.Ther.*, **5**, 1175-1182.
32. Hacein-Bey-Abina,S., Garrigue,A., Wang,G.P., Soulier,J., Lim,A., Morillon,E., Clappier,E.,

Caccavelli,L., Delabesse,E., Beldjord,K. *et al.* (2008) Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J.Clin.Invest*, **118**, 3132-3142.

33. Macpherson,J.L., Boyd,M.P., Arndt,A.J., Todd,A.V., Fanning,G.C., Ely,J.A., Elliott,F., Knop,A., Raponi,M., Murray,J. *et al.* (2005) Long-term survival and concomitant gene expression of ribozyme-transduced CD4+ T-lymphocytes in HIV-infected patients. *J.Gene Med.*, **7**, 552-564.
34. Amado,R.G., Mitsuyasu,R.T., Rosenblatt,J.D., Ngok,F.K., Bakker,A., Cole,S., Chorn,N., Lin,L.S., Bristol,G., Boyd,M.P. *et al.* (2004) Anti-human immunodeficiency virus hematopoietic progenitor cell-delivered ribozyme in a phase I study: myeloid and lymphoid reconstitution in human immunodeficiency virus type-1-infected patients. *Hum Gene Ther.*, **15**, 251-262.
35. Mitsuyasu,R.T., Merigan,T.C., Carr,A., Zack,J.A., Winters,M.A., Workman,C., Bloch,M., Lalezari,J., Becker,S., Thornton,L. *et al.* (2009) Phase 2 gene therapy trial of an anti-HIV ribozyme in autologous CD34(+) cells. *Nat.Med*, **15**, 285-292.
36. Levine,B.L., Humeau,L.M., Boyer,J., MacGregor,R.R., Rebello,T., Lu,X., Binder,G.K., Slepushkin,V., Lemiale,F., Mascola,J.R. *et al.* (2006) Gene transfer in humans using a conditionally replicating lentiviral vector. *Proc.Natl.Acad.Sci.USA*, **103**, 17372-17377.
37. Wang,G.P., Levine,B.L., Binder,G.K., Berry,C.C., Malani,N., McGarrity,G., Tebas,P., June,C.H. and Bushman,F.D. (2009) Analysis of lentiviral vector integration in HIV+ study subjects receiving autologous infusions of gene modified CD4+ T cells. *Mol.Ther.*, **17**, 844-850.
38. Li,M.J., Kim,J., Li,S., Zaia,J., Yee,J.K., Anderson,J., Akkina,R. and Rossi,J.J. (2005) Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. *Mol Ther.*, **12**, 900-909.
39. Ter Brake,O., Konstantinova,P., Ceylan,M. and Berkhout,B. (2006) Silencing of HIV-1 with RNA interference: a multiple shRNA approach. *Mol.Ther.*, **14**, 883-892.
40. Ter Brake,O., 't Hooft,K., Liu,Y.P., Centlivre,M., von Eije,K.J. and Berkhout,B. (2008) Lentiviral vector design for multiple shRNA expression and durable HIV-1 Inhibition. *Mol Ther.*, **16**, 557-564.
41. Banerjea,A., Li,M.J., Bauer,G., Remling,L., Lee,N.S., Rossi,J. and Akkina,R. (2003) Inhibition of HIV-1 by lentiviral vector-transduced siRNAs in T lymphocytes differentiated in SCID-hu mice and CD34+ progenitor cell-derived macrophages. *Mol.Ther.*, **8**, 62-71.
42. Chang,L.J., Liu,X. and He,J. (2005) Lentiviral siRNAs targeting multiple highly conserved RNA sequences of human immunodeficiency virus type 1. *Gene Ther.*, **12**, 1133-1144.
43. Ter Brake,O. and Berkhout,B. (2007) Lentiviral vectors that carry anti-HIV shRNAs: problems and solutions. *J Gene Med*, **9**, 743-750.
44. Kotsopoulou,E., Kim, V.N., Kingsman,A.J., Kingsman,S.M. and Mitrophanous,K.A. (2000) A Rev-independent human immunodeficiency virus type 1 (HIV-1)-based vector that exploits a codon-optimized HIV-1 gag-pol gene. *J.Virol.*, **74**, 4839-4852.
45. Westerhout,E.M., Ooms,M., Vink,M., Das,A.T. and Berkhout,B. (2005) HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Res.*, **33**, 796-804.
46. Westerhout,E.M. and Berkhout,B. (2007) A systematic analysis of the effect of target RNA structure on RNA interference. *Nucleic Acids Res.*, **35**, 4322-4330.
47. Schubert,S., Grunweller,A., Erdmann,V.A. and Kurreck,J. (2005) Local RNA target structure influences siRNA efficacy: systematic analysis of intentionally designed binding regions. *J.Mol. Biol.*, **348**, 883-893.
48. Brown,K.M., Chu,C.Y. and Rana,T.M. (2005) Target accessibility dictates the potency of human RISC. *Nat.Struct.Mol.Biol.*, **12**, 469-470.
49. Poluri,A. and Sutton,R.E. (2007) Titers of HIV-based vectors encoding shRNAs are reduced by a Dicer-dependent mechanism. *Mol Ther.*, **16**, 378-386.
50. Bridge,A.J., Pebernard,S., Ducraux,A., Nicoulaz,A.L. and Iggo,R. (2003) Induction of an interferon response by RNAi vectors in mammalian cells. *Nat.Genet.*, **34**, 263-264.

51. Sledz,C.A., Holko,M., de Veer,M.J., Silverman,R.H. and Williams,B.R. (2003) Activation of the interferon system by short-interfering RNAs. *Nat.Cell Biol*, **5**, 834-839.
52. Hornung,V., Guenthner-Biller,M., Bourquin,C., Ablasser,A., Schlee,M., Uematsu,S., Noronha,A., Manoharan,M., Akira,S., de,F.A. *et al.* (2005) Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat.Med.*, **11**, 263-270.
53. Bauer,M., Kinkl,N., Meixner,A., Kremmer,E., Riemenschneider,M., Forstl,H., Gasser,T. and Ueffing,M. (2009) Prevention of interferon-stimulated gene expression using microRNA-designed hairpins. *Gene Ther.*, **16**, 142-147.
54. Jackson,A.L. and Linsley,P.S. (2004) Noise amidst the silence: off-target effects of siRNAs? *Trends Genet.*, **20**, 521-524.
55. Grimm,D., Streetz,K.L., Jopling,C.L., Storm,T.A., Pandey,K., Davis,C.R., Marion,P., Salazar,F. and Kay,M.A. (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*, **441**, 537-541.
56. Silva,J.M., Li,M.Z., Chang,K., Ge,W., Golding,M.C., Rickles,R.J., Siolas,D., Hu,G., Paddison,P.J., Schlabach,M.R. *et al.* (2005) Second-generation shRNA libraries covering the mouse and human genomes. *Nat.Genet.*, **37**, 1281-1288.
57. Liu,Y.P., Haasnoot,J., Ter Brake,O., Berkhout,B. and Konstantinova,P. (2008) Inhibition of HIV-1 by multiple siRNAs expressed from a single microRNA polycistron. *Nucleic Acids Res*, **36**, 2811-2824.