RNA interference (RNAi) is a cellular mechanism that can be induced by small interfering RNAs (siRNAs) to mediate sequence-specific gene silencing by cleavage of the targeted messenger RNA. RNAi can be used as an antiviral approach to silence HIV type-1 (HIV-1) through stable expression of precursors, such as short hairpin RNAs (shRNAs), which are processed into siRNAs that can elicit degradation of HIV-1 RNAs. At the beginning of 2008, the first clinical trial using a lentivirus with an RNA-based gene therapy against HIV-1 was initiated. The antiviral molecules in this gene therapy consist of three RNA effectors, one of which triggers the RNAi pathway. This review article focuses on the basic principles of an RNAi-based gene therapy against HIV-1, including delivery methods, target selection, viral escape possibilities, systems for multiplexing siRNAs to achieve a durable therapy and the in vitro and in vivo test systems to evaluate the efficacy and safety of such a therapy.

After identification of HIV type-1 (HIV-1) in 1983 as a causative agent for AIDS [1,2], we have witnessed the endemic spread of this virus. AIDS is the major cause of death in the developing world and, worldwide, was responsible for 2 million deaths in 2007. An estimated 33 million people are infected with HIV-1 and the most affected countries are located in sub-Saharan Africa. A total of 2 million infected individuals are living in Western and Central Europe and North America. Up to now, efforts in developing a vaccine against HIV-1 have been unsuccessful. Since the introduction of highly active antiretroviral therapy, the quality of life and life expectancy of treated individuals in the Western world has improved markedly. However, strict drug regimens decrease the quality of life for most patients and low therapy adherence can result in the emergence of drug-resistant viruses and exhaustion of the current antiviral drug arsenal. In all, this warrants the development of new therapies that are preferably less dependent on patient compliance and with a durable effect and minimal side effects. A gene therapy might provide such an option for a specific group of HIV-1-infected individuals. The first clinical trial using a lentivirus with an RNA-based gene therapy against HIV-1 was initiated. The antiviral molecules in this gene therapy consist of three RNA effectors, one of which triggers the RNAi pathway. This review article focuses on the basic principles of an RNAi-based gene therapy against HIV-1, including delivery methods, target selection, viral escape possibilities, systems for multiplexing siRNAs to achieve a durable therapy and the in vitro and in vivo test systems to evaluate the efficacy and safety of such a therapy.

Using the RNAi pathway for therapeutic siRNA expression

The discovery of RNAi silencing in mammalian cells triggered the development of RNAi-based therapies against a wide variety of diseases, including cancer, neurological, autoimmune and infectious diseases [4–10]. The importance of RNAi in future drug development was underlined when the Nobel Prize in Medicine was awarded in 2006 to Andrew Fire and Craig Mello for the discovery of the RNAi mechanism [11]. RNAi also holds promise as a powerful strategy for intracellular therapy against pathogenic viruses, such as HIV-1. To properly evaluate RNAi therapeutic approaches and the risks involved in using the
cellular RNAi machinery it is essential to understand the natural RNAi mechanism and its function. Therefore, we will first describe the microRNA (miRNA) pathway in more detail. It is estimated that human cells can express more than 500 different miRNAs. miRNAs are important in cell regulation and development [12–18], and they regulate gene expression in humans by translational repression or messenger RNA (mRNA) cleavage and degradation [19].

Figure 1 depicts the natural miRNA pathway. Polymerase (Pol) II or III produces the primary transcript, pri-miRNA (miRNA), that encodes the miRNA. This pri-miRNA is processed into a pre-miRNA with a 5′ monophosphate (5′P) and a 2-nucleotide overhang with a 3′ hydroxyl (3′OH) end by Drosha and DGCR8/Pasha (depicted on the left). miRNAs present in introns (mirtrons) are also processed into pre-miRNAs through a distinct route using the splicing machinery (depicted on the right). Exportin-5 (Exp-5) exports the pre-miRNA from the nucleus to the cytoplasm. In the cytoplasm, Dicer binds to the pre-miRNA and cleaves the base-paired stem approximately 22 nucleotides away from its base, generating a 2-nucleotide overhang at the 3′ end. RNA-induced silencing complex (RISC) unwinds the miRNA and loads one RNA strand (guide strand) in the complex, the other strand gets degraded (passenger strand). When full complementarity is present between the messenger RNA (mRNA) and miRNA cleavage will occur, while translational repression occurs with near-perfect complementarity in the 3′ untranslated region (3′UTR) of the mRNA. TRBP, TAR RNA binding protein.

by a microprocessor containing the RNase III enzyme Drosha and a double-stranded RNA (dsRNA) binding protein termed DGCR8/Pasha [21–25]. miRNAs present in introns (mirtrons) are also processed into pre-miRNAs through a distinct route using the splicing machinery as depicted in Figure 1 [26,27]. The pre-miRNA is made in the nucleus and exported to the cytoplasm by Exportin-5 [28–30]. In the cytoplasm the RNase III endonuclease Dicer binds to the pre-miRNA and cleaves the base-paired stem approximately 22 nucleotides away from its base, generating a 2-nucleotide overhang at the 3′-end [31]. Dicer is associated with TAR RNA binding protein, which is required to recruit Argonaute 2 (Ago2) to the siRNA [32]. The Ago2–RNA complex forms the minimal core of the RNA-induced silencing complex (RISC) [33,34].
RISC unwinds the miRNA and loads one RNA strand (guide strand) in the complex; the other strand gets degraded (passenger strand) [35]. The RNA strand of the duplex with the lowest thermodynamic stability at its 5'-end gets preferentially incorporated into the complex [36,37].

In mammals, silencing is mainly elicited by translational repression of the targeted mRNA [19]. The most important determinant for mRNA cleavage or translational repression is target RNA recognition based on perfect or near-perfect complementarity of the miRNA with the mRNA, respectively [38–42]. Typically RISC forms complexes when the ‘seed’ region of the miRNA with the mRNA, respectively [38–42]. Typically RISC forms complexes when the ‘seed’ region of the miRNA (5'-end) finds multiple target sequences in the 3' untranslated region (3’UTR) of the mRNA. The number of targets in the 3’UTR and the distance between them has an effect on the silencing efficiency [43]. Mammalian miRNA base pairing occurs mainly by imperfect complementarity with the mRNA and translational repression, but at least one case of perfect complementarity and mRNA cleavage is known in humans [44]. Endonucleolytic cleavage of the targeted mRNA occurs opposite of nucleotide position 10 to 11 of the miRNA and the cleaved mRNA is subsequently degraded.

It is a rare event that natural miRNAs elicit mRNA cleavage in mammalian cells. However, artificial dsRNAs with full complementarity to an mRNA can direct mRNA cleavage, even with a single target site, which also does not necessarily have to be positioned in the 3’UTR. Artificial dsRNA can be produced by several methods, including synthetic mature siRNAs that need to be transfected into the cell [45] or short hairpin RNAs (shRNAs) [46,47] and artificial miRNAs that can be expressed intracellularly from a transgene construct [48]. Thus the natural miRNA pathway can be instructed for therapeutic downregulation of a specific mRNA. This therapeutic approach is interesting for diseases caused by the overexpression of a specific mRNA or to target the RNA genomes of invading microbes such as HIV-1.

Instruction of this cellular pathway with new siRNA specificity is associated with certain risks. One general risk factor is competition with the endogenous siRNAs and saturation of the miRNA pathway by the artificial siRNAs, shRNAs or miRNAs. Because the miRNA pathway is important in gene regulation, this can result in unwanted side effects such as cell death, disturbances in cell differentiation programmes or cancer. Several reports have shown that saturation of the miRNA pathway can occur and even lead to death when high doses of shRNAs were delivered by an Adeno-associated virus vector in mice [49–53]. Because miRNAs need only a seed sequence complementarity of 7–8 base pairs within the 3’UTR of an mRNA for function [54], there is a significant risk of targeting additional mRNAs, and such ‘off-target’ effects can be elicited by the passenger or guide strand [55–58]. Also the induction of an immune response by siRNAs and shRNAs has been reported previously [59,60], but this can be avoided by optimal design of the siRNA/shRNA molecule [61].

The basic principles of an RNAi-based gene therapy against HIV-1

The main focus of an RNAi-based gene therapy approach against AIDS would be the protection of HIV-1-susceptible cells of the immune system, being the CD4+ T-cells, monocytes, macrophages and dendritic cells. This ‘intracellular immunization’ will prevent the depletion of these immune cells during disease progression. Maintenance of the immune system should prevent opportunistic infections and progression towards AIDS. HIV-1 causes a chronic infection and no viral clearance occurs; therefore, continuous treatment is required. Repeated delivery of exogenous siRNAs as anti-HIV-1 therapy has been described [62] in a humanized immune system (HIS) mouse model. This study showed effective virus inhibition and the loss of CD4+ T-cells was prevented. However, it is doubtful if such an approach would be suitable in a patient setting, where the prevention of viral escape requires the continuous presence of an effective dose of siRNA in all infected human cells that are present in many different body compartments. Thus, we strongly prefer the concept of continuous expression of anti-HIV-1 molecules obtained by a single transduction with a lentiviral vector. A lentiviral vector is derived from the HIV-1 virus itself. The pathogenic genes are replaced by novel control and therapeutic sequences. The lentiviral vector does infect the target cell and deposits the transgene, but it cannot replicate. The benefit of a lentiviral vector compared with other viral delivery methods is that it can transduce dividing and non-dividing cell types and that it stably transduces cells because the vector is integrated into the genome [63].

Figure 2 depicts the gene therapy procedure, including the lentiviral vector production scheme. Several studies indicate that haematopoietic stem cells (HSCs) are not susceptible to HIV-1 infection [64,65]. Because they seed the different lineages of immune cells in the blood, they form interesting candidates for the gene therapy, followed by autologue transplantation of these lentiviral-vector-treated HSCs. The therapeutic lentiviral vector will equip all derived immune cells with the antiviral arsenal. In addition, preferential outgrowth of the shRNA-expressing immune cells over untreated cells under HIV-1 pressure would result in an increase in the percentage of protected immune cells. Thus, the treatment should result in a partial or complete
reconstitution of the immune system, preventing HIV-1 infection to progress towards AIDS. In an ideal setting, the treatment of a patient with a single gene therapy would achieve a durable effect. Clinical trials with HSCs that were transduced with retroviral-delivered anti-HIV-1 ribozymes have previously been performed [66–68]. These trials demonstrate the feasibility of the proposed stem cell approach. Another option is the treatment of the mature CD4+ T-cell population, in which case repetitive gene therapy should be applied.

**Figure 2. Lentiviral vector production and clinical approach**

A HIV-1-positive patient failing on regular HAART drug therapy

B Lentiviral vector production

C Apheresis stem cell/T-cell enrichment

D Transduction target cells

E Infusion of treated cells

F Protected immune cells prevents progression to AIDS

In a therapeutic setting, an HIV type-1 (HIV-1)-positive patient failing on regular therapy (A) could be treated with the RNA-interference-based lentiviral vector. For lentivirus production (B) lentiviral vector (JS1) and packaging plasmids (pRSV-rev, pVSV-g and pSYNGP) are transfected in the 293T producer cells. The lentiviral vector will produce viral genomes and the packaging plasmids will produce the proteins required to form the viral particle. The pRSV-rev produces the Rev protein important for export of full genomes out of the nucleus, the pVSV-g produces the vesicular stomatitis virus glycoprotein that is used for virus pseudotyping and the pSYNGP plasmid produces the Gag and Pol proteins. At 48 or 72 h post-transfection, virus particles are collected, concentrated and purified for the transduction of the target cells. The patient would undergo an apheresis for the collection of stem cells or T-cells (C). After enrichment for the target cells transduction with the therapeutic construct would be performed (D). With this transduction, cells will get a stable integration of the short hairpin RNA (shRNA) therapy, this 'intracellular immunization' will protect these cells against HIV-1. Treated cells will be infused into the patient (E) and the protected immune cells will hopefully prevent the disease progression towards AIDS (F). HAART, highly active antiretroviral therapy; mRNA, messenger RNA; RISC, RNA-induced silencing complex; siRNA, small interfering RNA.
because T-cells have only a limited life span [69]. By contrast, the transduced stem cells should continue to generate immune cells of different lineages.

**Therapeutic vector design**

Potent and sequence-specific HIV-1 inhibition has been reported with RNAi-inducing reagents in cell culture infections. It soon became apparent that HIV-1 is prone to viral escape in a mono-shRNA therapy [70–78], similar to single antiretroviral drug regimens. The therapeutic vector used in a clinical trial should therefore always tackle the virus with multiple inhibitors at the same time. This can be a combination of shRNAs that target the virus [79] or host-encoded cofactors, or both. One could also combine RNAi molecules with other RNA effector molecules, such as decoys and ribozymes [80]. Another elegant solution to avoid viral escape is the use of second-generation shRNAs that specifically target viral escape variants [81]. However, the relatively high number of viral escape routes available to HIV-1 reduces the feasibility of this approach [78].

We currently consider the *ex vivo* delivery approach of a lentiviral therapeutic vector the most suitable for HIV-1 treatment. Some problems can be encountered when using lentiviruses to target HIV-1 with RNAi, such as self-targeting of the transgene RNA by the shRNA expressed in the producer cell and targeting of HIV-1-derived sequences in the vector genome. These problems and solutions have previously been discussed in great detail by ter Brake and Berkhout [82]. Besides lentiviruses, there are other vectors available for delivering a therapeutic RNAi transgene and these have been extensively discussed by others [83–85]. In this review, we address the important issues concerning therapeutic vector design against HIV-1.

**Selecting the optimal viral target sites**

Firstly, a combination of potent inhibitors has to be assembled. Several criteria can be formulated for the identification of optimal target sites for RNAi attack on the 9 kb HIV-1 RNA genome. One option is the selection of target sites in the early spliced mRNAs encoding the early proteins (Tat, Rev and Nef). By an early block in viral gene expression, the expression of the late structural proteins and virion assembly will be severely affected. Also the selection of regions that are present in all mRNAs, both the spliced and unspliced RNA forms, could be a good strategy. Such regions are present in the untranslated leader RNA and the 3’-terminal *nef* gene. Stability of the target RNA structure can affect the RNAi silencing effect, as highly structured regions are inaccessible for RISC [76,86]. Thus, targeting of ‘open’ regions of the genome might be beneficial. In addition, the selection of highly conserved regions, allowing inhibition of as many virus strains as possible, is an important selection criteria. As an important bonus, targeting of highly conserved regions might prevent the easy generation of escape mutants. An extensive screen with shRNAs targeting the most conserved sequences of the HIV-1 genome has been performed, yielding approximately 20 candidate shRNAs [74]. We and others have identified effective shRNAs and siRNAs targeting the regulatory long terminal repeat (LTR) sequence [74,87] and almost all HIV-1 genes: *gag* [88–91], *pol* [74,88,89,92], *vif* [87], *tat* [74,92–94], *rev* [74,93,94], *vpu* [88], *env* [91] and *nef* [87]. A sensitive screen to select the most potent shRNAs should include extended culturing of stably transduced T-cells with and without HIV-1 to score the effect on cell viability and efficacy of HIV-1 suppression. Even for the most potent inhibitors, there is a possibility that escape occurs over time. An escape analysis of the virus can be performed to verify the selective pressure put on the virus by the shRNA. The appearance of mutations in the viral target sequence forms the dramatic demonstration of the sequence-specificity of RNAi inhibition. When only wild-type sequences are observed in breakthrough viruses, this is in fact an indication of weak inhibition [78,95]. To address the safety, targeting of human mRNAs by the antiviral shRNAs can be evaluated [55]. We have recently described guidelines for testing of shRNA inhibitors against HIV-1 [95].

**Cellular target selection**

An advantage of targeting host cell factors that are important for HIV-1 replication is the reduced change of viral escape. Factors that have been downregulated with shRNAs to successfully prevent HIV-1 replication include nuclear factor-κB [92], CD4 [90], CXCR4 [96], DDX-3 [97], LEDGF/p75 [98] and CCR5 [99].

CCR5 is a promising and well-studied target. Humans that are homozygote for the Δ32 mutation in this gene do not get infected with HIV-1 because CCR5 is a crucial receptor for HIV-1 cell entry. These individuals appear healthy, possibly with an increased risk for infection with the West Nile virus [100]. A potent shRNA targeting this host cell factor has been developed [101,102]. Although CCR5-tropic viruses are generally responsible for transmission to other individuals, CXCR4-tropic viruses can evolve in HIV-1-infected individuals. When the CCR5 receptor is downregulated, this will potentially select for CXCR4 viruses. Whether this selection will be induced upon shRNA-silencing of CCR5 remains unknown. Many targets will obviously not be good candidates for a gene therapy because they are essential for the cell. For example, CXCR4 is
required for homing of the HSCs to the bone marrow and subsequent T-cell differentiation [103].

High-throughput RNAi gene knockdown screens have recently been performed that describe many candidate host factors that are essential for HIV-1 replication [104–106]. These studies surprisingly did not point to the same set of cofactors. These cofactors should first be validated in other assays to exclude false-positives. Such studies will hopefully increase the arsenal of cellular targets available for a combinatorial shRNA approach.

Other RNA effector molecules

Other types of inhibitory RNA molecules that target the virus can also be used. A combination of several types of antiviral RNA molecules is used in the ongoing Phase I clinical trial at the City of Hope National Medical Center. The lentiviral vector that is used encodes a TAR-decoy, CCR5 ribozyme and a shRNA targeting the viral genome in the tat–rev region [80]. The TAR-decoy is a small nucleolar RNA molecule that binds Tat, which will prevent the Tat–TAR interaction that is essential for enhanced viral promoter activity [107]. The CCR5 ribozyme cleaves the CCR5 mRNA causing mRNA degradation and a reduction of CCR5 expression on the cell surface [108]. Comprehensive reviews on combinatorial approaches are available [109,110]. Some examples of other anti-HIV-1 RNA molecules are antisense transcripts [111,112], decoys [107,113], ribozymes [108] and aptamers [114]. A new addition to this arsenal is an antisense molecule that can elicit transcriptional gene silencing at the viral LTR promoter [115]. Another novel technique is RNAu, which is based on expression of a modified U1 small nuclear RNA (snRNA) [116]. The 5′ nucleotides 2–11 of U1 are replaced to allow base pairing with a 10-nucleotide target in the 3′-terminal exon of the gene of interest. Inhibition results from blocking polyadenylation upon binding of the modified U1 snRNA, followed by degradation of the RNA transcript.

Multiplexing siRNAs

After the selection of the most optimal combination of effector molecules, the second step is to choose the expression method to multiplex several RNA inhibitors in a single therapeutic vector. A variety of strategies have been described for multiplexing shRNAs that inhibit HIV-1 replication (Figure 3). Simultaneous expression of multiple shRNAs can be achieved from separate Pol III promoters or a combination of Pol II and III promoters [79]. Alternatively, one can design extended shRNAs that will be processed into two or three siRNAs [117] or long hairpin RNAs that should encode many siRNAs [118–120]. The disadvantage of the latter approach is that it is unclear up front whether the produced siRNAs will be active inhibitors [81]. Pol II expressed polycistronic miRNA clusters have also been developed [121,122], which can be combined with a TAR-decoy [121].

An important aspect when considering the expression of multiple siRNAs is to achieve equimolarity of the siRNAs, as unequal pressure on different targets might allow viral escape. The total siRNA expression level is also important. It should suffice to obtain efficient viral inhibition, without interfering with the natural miRNA pathway. Although various groups have reported toxicity of shRNA [49–53], this could be solved by inserting the siRNA into a natural miRNA backbone [50].

Conditional expression of the siRNA molecules could potentially increase the safety of a therapeutic vector. It would be particularly beneficial to avoid shRNA expression in the HSCs that still have to undergo haematopoiesis, a process that might be particularly sensitive to changes in the RNAi machinery. Tissue-specific miRNA expression has been described for the liver [123]. Another option is the design of constructs that are induced by HIV-1 infection [75]. Selective expression in HIV-1 susceptible cells would be an elegant way to restrict putative saturation and off-target effects. Another option is the use of drug-inducible gene expression systems, such as the doxycycline-dependent Tet system [124,125], but it is questionable if this system will suffice for a clinical application against HIV-1. shRNAs are mainly expressed from Pol III promoters, but on some occasions also from modified Pol II promoters (U1). The miRNAs are mostly expressed from Pol II promoters. For tissue-specific or drug-regulated expression, the Pol II systems are better equipped. The need for conditional expression of an RNAi-based gene therapy against HIV-1 has to be addressed in further detail.

Evaluation of the safety and efficacy of an RNAi-based gene therapy

Once potent antiviral shRNAs are successfully expressed from a single vector, it is necessary to move the relevant preclinical models to critically assess the safety and efficacy. An elegant and sensitive method to measure the effect on cell viability in vitro is to perform a coculture of green fluorescent protein (GFP)-positive transduced cells and non-treated cells (unpublished results). A reduction over time in the percentage of GFP-positive cells is an indication of delayed cell growth and toxicity. To address the efficacy, outgrowth of the transduced cells can be monitored under HIV-1 pressure, again using GFP analysis by FACS as a simple marker of the composition of the cell mixture. Of course, one can
also perform virus replication studies in pure cultures of protected versus control cells.

The next stage requires appropriate in vivo models. The simian immunodeficiency virus (SIV)/macaque model [126] has been used extensively for vaccine studies. This model could also be considered for testing of an anti-HIV-1 RNAi gene therapy, but it has several limitations. First, anti-HIV shRNAs cannot easily be tested against SIV because of sequence dissimilarity, and the same probably holds for host genes from human versus macaque. Thus, the anti-HIV shRNAs should either be converted into anti-SIV shRNAs, which might affect their inhibitory power, or HIV-1 target sequences can be incorporated into the SIV genome. Second, transduction of the HIV-1-based lentiviral vector is restricted by TRIM5α in macaque cells [127]. Third, macaque experiments are rather expensive, and the number of animals that can be used is restricted. Recently, a minimally modified simian-tropic HIV-1 strain has been developed that infects lymphocytes and causes an acute viraemia and persistent infection for several months in pig-tailed macaques [128]. In contrast to most infected humans, simian tropic HIV-1 infection gets controlled in the macaque model after several months. Most of these limitations do not apply to HIS mouse models [129,130]. In the most recent HIS mouse model, all major human myeloid and lymphoid cellular compartments develop and mature from input human stem cells [131–133]. This model provides access to in vivo and ex vivo experimentation on human T-cells [134]. HIS

Figure 3. Strategies for multiplexing siRNAs

Simultaneous expression of multiple short hairpin RNAs (shRNAs) can be achieved by several strategies depicted from top to bottom: (A) Expression from separate polymerase (Pol) III promoters, (B) a combination of Pol II and III promoters, (C) long hairpin RNAs, (D) extended shRNAs, (E) Pol II expressed polycistronic microRNA cluster and (F) a Pol II expressed polycistronic microRNA cluster combined with a TAR-decoy (TARd). CMV, cytomegalovirus; siRNA, small interfering RNA.
mice can be infected through injection of the virus, but also through rectal and vaginal transmission routes. Infection results in viraemia and the depletion of human CD4+ T-cells as seen in the disease course of infected patients [135–140]. We used this model to test the safety and efficacy of a lentiviral-based gene therapy of stem cells using a shRNA targeting the Nef region [10]. These mouse models and other animal models, including the advantages and limitations, have been discussed previously in a report written by several experts [141].

What can be learned from previous and ongoing clinical trials?

In 1990, the first patient suffering from adenosine deaminase deficiency – a form of severe combined immunodeficiency (SCID) – was treated with a gene therapy [142]. In 1999, the first report was made public of a patient who died because of the administration of a gene therapy. This patient was treated for a genetic liver disease (ornithine transcarbamylase deficiency) and received an adeno virus treatment with the wild-type gene. He died 4 days later of a massive immune response, most likely triggered by the use of the viral vector [143]. In 2000, a trial in which patients with SCID received a γ-retroviral gene transfer with the wild-type interleukin-2 gene started. Although this procedure was a true success and improved the condition of all the patients [144], two patients developed a leukaemia-like condition of clonal lymphocyte proliferation. Both cases were caused by γ-retrovirus vector integration in or near the promoter of the LMO2 proto-oncogene, leading to enhanced expression of the LMO2 protein, which has a crucial role in haematopoietic development [145]. After this initial report, more patients in this and a similar trial developed leukaemia-like conditions. By now more than 1,300 clinical trials involving a gene therapy have been performed [146]. From these clinical trials, lessons can be learned for future improvement and development of gene therapies. Retroviral vectors have in general been replaced by lentiviral vectors, which are much more efficient and safe because all transcription enhancer motifs have been removed and because these vectors tend to integrate in genes, and not near the promoter region.

Therapeutic benefit

A review that provides an overview of gene therapy trials for HIV-1 is available [147]. Although positive results were obtained in vitro for these HIV-1 gene therapies, the clinical trials failed to demonstrate clear evidence of therapeutic benefits. In studies where T-cells or HSCs were treated with γ-retroviral vectors, one of the limitations was effective gene delivery to a clinically relevant number of cells that persist after reinfusion [68]. Lentiviral vectors will be much more efficient in this respect. In addition, many of the previously used RNA molecules did not harbour very potent inhibitory capacities. For a sustainable HIV-1 inhibition, viral replication should be blocked almost completely. With optimized shRNAs, a complete inhibition of virus replication can be achieved with only a single inhibitor expressed from a single integrated lentiviral vector. RNAi is therefore a promising candidate for a future anti-HIV-1 gene therapy.

The requirement to obtain a sufficient number of protected cells might be met by developments in viral vector technology. The lentiviral vector has a better capacity to transduce T-cells and HSCs compared with the previous γ-retroviral vectors. Also, improved vector production methods can increase the transduction efficiency in a therapeutic setting. The first clinical trial with a lentiviral vector was indeed an anti-HIV-1 gene therapy that uses an antisense molecule that is expressed in T-cells. Persistent in vivo expression of the therapeutic antisense molecule was observed [112]. In addition, vector integration sites in the blood cells revealed a preference for gene-rich regions, which is typical for a lentivirus, and no evidence of insertional oncogenesis was observed. A trial was started recently in which children with X-linked adrenoleukodystrophy are treated with ex vivo-modified HSCs using a lentiviral vector. Promising results were presented by Cartier-Lacave [148] at the ESGCT 2008 meeting in Bruges, Belgium demonstrating highly effective transduction, persistence of the treated cells and clinical improvement. The clinical trial with the triple antiviral approach and a lentiviral vector at the City of Hope National Medical Center also shows promising results. To date, four patients have been treated and all are doing well. Persistent gene marking and consistent shRNA expression levels are detected in the peripheral blood mononuclear cells of all patients, and the first patient has been followed for a period of 8 months (J Rossi, City of Hope National Medical Center, personal communication). Overall, these results are promising for the potential of reaching therapeutic benefits with future gene therapy protocols against HIV-1.

Safety of lentiviral vectors

Similar to γ-retroviral vectors, lentiviruses integrate in the human genome; therefore, potential insertional oncogenesis remains a matter of concern. Lentiviral vectors are derived from HIV-1, thus we could argue that these vectors do not provide an additional risk of insertional oncogenesis in HIV-1-infected individuals. In fact, no cases of cancer induced by the integration of HIV-1 have been described, but these cells might have been removed.
by the activated immune system. One could argue that the protected cells will survive, possibly leading to re-establishment of the oncogenic threat. Also, in vivo experiments with a lentiviral vector in HSCs in tumour-prone mice did not, in contrast to the γ-retrovirus, show signs of insertional oncogenesis [149].

This difference in tumour induction capacity can be explained by differences in integration site preference and the presence of enhancer elements in the γ-retroviral vector. The lentivirus targets gene-rich regions at a higher frequency than γ-retroviruses, but without the predilection for the 5'-end of the gene. Viral insertion near the 5'-end of a gene and the presence of strong enhancer elements in the γ-retroviral LTRs can transactivate the adjacent cellular gene promoter and cause insertional mutagenesis [150]. Most lentiviral vectors have the so-called self-inactivating (SIN) LTR elements. Such SIN vectors have a lower propensity to transactivate adjacent genes than γ-retroviral vectors [149]. However, insertional mutagenesis is something that has to be tested for each combination of vector and therapeutic gene insert. Other safety and regulatory issues concerning lentiviral vectors are addressed in a comprehensive review based on the expertise gained in the first lentiviral trial [151].

Ensure sequence-specific effects of the therapeutic effector molecule

An important lesson that can be learned from various siRNA clinical trials concerns the selection of the correct controls. In several studies, a control siRNA was used that targets GFP. These studies focused on the inhibition of infections and inflammation. Results were in favour of a therapeutic effect, but later it became evident that the GFP siRNA control is particular in being of low immunogenicity compared with other shRNAs, including the therapeutic ones. Thus, the therapeutic effect was not elicited by specific downregulation of the target mRNA. Most siRNAs trigger the TLR7/8 interferon pathway, but the less immunogenic GFP siRNA control did not [152].

Another lesson comes from a study on an siRNA therapeutic designed for the eye against blinding choriodal neovascularisation, a form of age-related macular degeneration. The siRNA caused improvement of the disease, but this effect was not elicited by the RNAi mechanism because the effector molecule cannot penetrate the cell. Instead, the clinical effect was obtained through Toll-like receptor-3 signalling [153]. Both examples illustrate the importance of selecting the correct controls and to determine the correct endpoint of a study to ensure that one is looking at RNAi-specific effects of the siRNA/shRNA. For HIV-1 therapies that target the viral genome, exclusive specificity can be demonstrated with HIV-1 variants that have one or more point mutations that render the virus RNAi resistant. With this information, the sequence specificity of a particular siRNA effector molecule can be demonstrated [10,78,79]. This approach could also be suitable for animal models, before moving to the clinical phase.

Future perspectives

In this review we provide an overview of the current status of the development of an RNAi-based gene therapy against HIV-1/AIDS, thereby focusing on the possibilities, concerns and limitations. Overall, an RNAi-based gene therapy against HIV-1 seems to be a promising candidate for a durable antiviral treatment, especially for a minority patient group for which the treatment options are exhausted. The potent and specific viral inhibition obtained with RNAi, the transduction potency of lentiviral vectors in combination with HSCs and the availability of standard lentiviral production facilities are promising developments in the field. We are currently testing a candidate clinical vector containing four shRNAs to evaluate safety and efficacy. In vitro this vector gave promising results, yielding very potent antiviral effects in long-term cultures [79]. Hopefully safety and efficacy will be demonstrated in future experiments in the humanized mouse model, such that a clinical trial can be initiated within 2 years.

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