RNAi based gene therapy for HIV-1, from bench to bedside
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After identification of the human immunodeficiency virus type 1 (HIV-1) in 1983 as the causative agent for the acquired immunodeficiency syndrome (AIDS) [26, 97], we have witnessed the pandemic spread of this virus. AIDS is the major cause of death in the developing world and was worldwide 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. Approximately two million infected individuals are living in Western and Central Europe and North America [297]. Up to now, efforts in developing a vaccine against HIV-1 have been unsuccessful.

Since the introduction of highly active anti-retroviral therapy (HAART), the quality of life and life expectancy of treated individuals in the Western world has improved dramatically. However, strict drug regimens and drug side-effects 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 pleads for the development of new therapies that are preferably less dependent on patient compliance, with a durable impact and minimal side effects. A gene therapy could provide such an option for a specific group of HIV-1 infected individuals. The first clinical trial using a gene therapy based on RNA interference (RNAi) and other antiviral RNA molecules (TAR-decoy and CCR5-ribozyme) has started in the United States in the City of Hope National Medical Center in 2008 [57]. In this chapter I will give a general overview of HIV-1 and its replication cycle, the current treatment options, followed by an introduction on the RNAi mechanism. I will subsequently focus on the basic principles of an RNAi-based gene therapy against HIV-1.
1.1 HIV-1

HIV-1 belongs to the *lentivirus* genus, a subfamily of the *Retroviridae*. All retroviruses carry a diploid positive stranded RNA genome that is reverse transcribed into a double-stranded (ds) DNA copy that integrates into the host cell genome. The proviral DNA genome is approximately 9.5 kb in size and contains 9 genes [239, 310]. Five viral genes (*gag, pol, env, tat* and *rev*) encode proteins that are essential for replication and the accessory genes (*vif, vpr, vpu* and *nef*) encode proteins that are needed for efficient virus spread and pathogenesis *in vivo*, but that are non-essential in some cell culture systems [91, 100, 239, 255, 274, 296, 310]. The role of these accessory proteins appears complex and multi-facetted, for instance the evasion from and manipulation of adaptive and innate immunity. For more details on the individual roles of these proteins see reviews [91, 195]. Besides the coding regions, the proviral DNA genome contains the long terminal repeats (LTRs) at the termini that consist of the U3 (unique 3'), R (repeat region) and U5 (unique 5') domains. The 5' LTR encodes the transcriptional promoter and other structure and sequence motifs that are important for viral replication [36, 98].

Figure 1.1 depicts the complete DNA viral genome and the different RNA splice variants. Transcription of the genome starts at the U3-R border in the 5' LTR and is terminated by polyadenylation at the R-U5 border in the 3' LTR. A complete proviral DNA genome is regenerated from this RNA transcript in the complex process of reverse transcription. HIV-1 has a large number of competing splice donor and acceptor sites that give rise to more than 40 differentially spliced HIV-1 mRNAs that can be divided into three main classes: the multiple spliced, single spliced and unspliced genomic RNAs [237]. This alternative splicing enables the production of 9 different proteins from a single primary RNA transcript.

The *gag* open reading frame encodes the Gag polyprotein that is proteolytically processed by Protease (PR) into the structural proteins Matrix (MA), Capsid (CA) and Nucleocapsid (NC) during particle assembly [39]. The *pol* open reading frame encodes the Gag-Pol polyprotein precursor that is post-translationally cleaved to produce the viral protease (PR), reverse transcriptase (RT) and integrase (IN) enzymes. The Gag-Pol polyprotein is generated by a ribosomal frameshift to the -1 frame while translating the C-terminal end of *gag* [280]. The envelope glycoproteins, surface protein SU-gp120 and the transmembrane subunit TM-gp41 are encoded by the *env* open reading frame. The Tat protein, important for transcriptional activation is encoded by the *tat* gene, and the *rev* gene encodes the Rev protein that is important for recruitment of nuclear export factors to facilitate nuclear export of unspliced and singly spliced transcripts as mentioned above [235]. In the late phase of viral RNA production, single spliced and unspliced mRNAs are produced that contain the Rev Responsive Element (RRE), this RNA structure within the *env* open reading frame binds Rev to promote nuclear export of these transcripts.

PR is released from the polyprotein by autoproteolysis at a late stage of viral assembly, which ensures that Gag proteins are not processed before they assemble. Gag processing by the viral PR initiates the process of virion maturation. After
1.1. HIV-1

HIV-1 DNA genome

HIV-1 mRNA splice variants

Figure 1.1: The DNA genome of HIV-1 and the RNA splice classes. The HIV-1 DNA genome is depicted. The genome contains an LTR at the termini, this LTR consist of the U3, R and U5 region. Also the reading frames for the 9 proteins are depicted. The 5′ LTR acts as a promoter for the synthesis of viral RNA. Below the different mRNA splice variants are depicted: the multiple spliced, single spliced and full genome transcripts. RNA sizes are listed at the left and at the right the encoded proteins are listed.
assembly, budding and maturation, the viral particle is ready to infect new host cells. Figure 1.2 depicts the viral particle with its essential components.

**Figure 1.2: HIV-1 virus particle.** The core of the viral particle contains the viral RNA genome that is present as a dimer and associated with the viral nucleocapsid protein (NC). This core is embedded in the virion capsid structure together with the Pol-encoded proteins Integrase (IN), Reverse Transcriptase (RT) and Protease (PR). The cone-shaped virion capsid is formed by the Gag encoded capsid proteins (CA). The Gag-encoded matrix protein (MA) lines the inside of the membrane. The viral particle is enveloped by a lipid membrane that contains the viral Env protein, which consist of the TM-gp41 and SU-gp120 subunits. See text for further details.

The retroviral life cycle is depicted in Figure 1.3. HIV-1 attaches to the target cell via its envelope protein SU-gp120 that first binds to CD4, followed by binding to a co-receptor (CCR5 or CXCR4). This elicits fusion of the viral and cellular membrane, triggering release of the viral core into the cytoplasm. In the core viral RNA is reverse transcribed by the RT enzyme into DNA, and the IN enzyme inserts the viral DNA genome into the host genome. Inserted viral DNA serves as a template for the production of viral mRNA, including new viral RNA genomes. In the last phase before leaving the cell the viral proteins and two RNA genome copies form a virus particle that leaves the cell by budding. After maturation the viral replication cycle is complete and the viral particle can infect a new target cell. For a more detailed description several reviews are available [105, 191].

HIV-1 causes a chronic infection that is characterized by three phases: acute infection, clinical latency and disease progression [285]. HIV-1 infection has been reported in various nonlymphoid cells including endothelial and epithelial cells [67, 327], but the majority of infections occur in a subset of immune cells (CD4-positive T cells, monocytes, macrophages and dendritic cells) and this eventually results in the collapse of the immune system and thus AIDS. This immune-compromised state triggers opportunistic infections and certain types of cancer and will result in death when untreated. Viral replication can be inhibited with antiviral drugs, but there is currently no cure available that can eliminate the virus. Thus prevention should remain a key factor in
Figure 1.3: Viral life cycle. The virus replication steps inhibited by the current drugs are depicted with letters: (A) entry, (B) reverse transcription, (C) integration and (D) maturation. See text for further details.

an attempt to control the pandemic, with additional measures such as safe sex education and needle exchange programs, but more drastic methods such as circumcision could be considered [66]. In several countries success in reducing the HIV prevalence has been obtained. However, these prevention campaigns have not been able to stop viral spread in the population. The difficulties to obtain success with these campaigns lay in the challenge to maintain continued awareness, effective communication of the prevention methods and the true availability of preventive measures such as condoms. Thus, efforts to develop an effective HIV-1 vaccine should continue apace.
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A entry

**CD4 binding**

- CD4 inhibitors

**co-receptor binding**

- CCR5/CXCR4 inhibitors

**virus cell fusion**

- Fusion inhibitors

B reverse transcription

**RT**

**NRTI**

**NNRTI**

C integration

**viral cDNA**

- Viral integrase

**Cell genome**

- Strand transfer inhibitors

D maturation

**polyprotein**

- MA CA NC p6

**protease inhibitors**

- Specific cleavage inhibitor

Figure 1.4: Treatment options for HIV-1. Drugs inhibiting HIV-1 at various steps in the viral life cycle have been developed. The first step in viral replication that can be blocked is entry (A). Viral entry goes through different virus-cell interactions, first the viral particle binds to CD4 with the gp120 domain, no drugs blocking this step are available. Secondly co-receptor binding occurs; two coreceptors can be used for this interaction, CCR5 and CXCR4. Currently one CCR5 blocker is available on the market (maraviroc). CXCR4 inhibitors have been developed, but they induced severe adverse effects. Thirdly, gp41/gp120 undergoes a conformational change leading to fusion of the virus with the cell. Drugs that bind to these domains can prevent the conformational change and thus virus-cell fusion, an example of such a drug is enfuvirtide that binds to gp41.

Another step that can be blocked in the viral life cycle is reverse transcription (B). Nucleoside reverse transcription inhibitors (NRTIs), such as zidovudine, block reverse transcription by acting as nucleoside analogues that cause chain termination. The non-nucleoside reverse transcription inhibitors (NNRTIs) inhibit HIV-1 by binding/blocking the RT enzyme; an example is nevirapine. Once viral cDNA is made, integration can be blocked (C) by strand transfer inhibitors that prevent incorporation of the viral cDNA into the host cell genome; for example the drug raltegravir.

Maturation of the virus particle is the last step that can be inhibited successfully (D). Protease inhibitors (PI, e.g. saquinavir) prevent the cleavage of the HIV polyprotein in the immature virus particle, which prevents maturation of the virus particle.

1.2 Current HIV-1 treatment

After virus exposure the establishment of an infection can be prevented in some cases by immediate prophylactic treatment with anti-viral drugs (PEP-prophylaxis) [III]. After HIV-1 diagnosis, HAART is advised when the CD4 cell count drops below the threshold of 350 cells/mm³ [I]. When disease progression results in AIDS, additional drugs treating the opportunistic infections and cancers can be added to the patients drug regimen. The first anti-HIV-1 drug zidovudine belonging to the NRTI group was approved in 1987 by the Food and Drug Administration (FDA) in the USA
and in the subsequent years other drugs followed. Currently, the following groups of antiviral drugs are available: NRTI, PI, NNRTI, entry inhibitors (fusion inhibitors and the CCR5 antagonist) and integrase inhibitors. A new maturation inhibitor is currently being tested in a phase II clinical trial. This drug, bevirimat, specifically disrupts cleavage of the CA-SP1 cleavage site in the Gag precursor protein [181]. Treatment depends on a patient’s individual situation, such as drug side-effects and viral resistance patterns. But the standard treatment often starts with a combination of two NRTI and one PI or NNRTI drug [117]. Atripla, a combination of two NRTIs and one NNRTI is available as a one pill daily drug regimen. Thus, while there are several classes of drugs available, the backbone of HIV treatment still consist of the well studied (N)NRTI and PI drugs. In Figure 1.3 the point of inhibition in the viral life cycle is indicated for the available drugs. Mechanisms of action are depicted and discussed in Figure 1.4.

In 1996, annual AIDS deaths in the United States decreased for the first time since the start of the pandemic. However, quality of life of the surviving patients was impacted due to side effects and demanding drug regimens. Side effects range from milder symptoms such as nausea, fatigue and diarrhea to problems with fat metabolism and fatal organ failure [54, 55]. These problems can lead to suboptimal therapy adherence (compliance). In turn, low patient compliance, combined with the high replication rate and error-prone replication machinery of HIV-1 can result in the emergence of drug resistance [24, 29, 30, 31, 38, 124, 132]. The presence of drug-resistant virus strains limits future therapy options, which leads to a worsened prognosis [133]. A related concern is the transmission of such drug-resistant virus strains to other individuals. Even if treatment results in long-term suppression of HIV-1 production, as evidenced by an undetectable HIV RNA plasma level, there is evidence that virus inhibition is not complete and that a low level of HIV replication remains [304, 336]. In all, these data underline the importance of a continued search for new therapeutic options, which include the development of a durable RNAi gene therapy against HIV-1 in which patient compliance does not play such a pivotal role.

### 1.3 The discovery of RNA interference

RNA interference (RNAi) is a cellular mechanism that can be induced by small interfering RNAs (siRNAs) to mediate sequence-specific gene silencing by cleavage or translational repression of the targeted mRNA. The observed phenomenon that led to the discovery of RNAi was made by Jorgensen and colleagues in petunia flowers that turned partially or completely white after the introduction of a pigment-producing gene that was supposed to deepen the purple color [218]. Around the same time, van der Krol observed similar results in petunias [301]. Soon similar phenomena were described in the fungi *neurospora crassa* by Romano and Macino, where this post transcriptional gene silencing (PTGS) is known as quelling [246]. Fire and Mello described the RNA interference phenomenon in the nematode *Caenorhabditis elegans*. Upon introduction of dsRNA, efficient sequence-specific gene silencing was
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observed \[95\]. Injection of dsRNA corresponding to different genes in the *Drosophila melanogaster* resulted in null mutant genotypes for the specific gene \[149, 211\]. Subsequently, RNAi was also observed in *Trypanosoma brucei* \[219\], zebrafish \[311\] and the mouse \[320\]. Introduction of base paired 21-nucleotide dsRNA into mammalian cell lines led to sequence-specific gene silencing \[90\]. This discovery triggered the development of RNAi-based therapies against a wide variety of diseases, including cancer, neurological, autoimmune and infectious diseases \[23, 71, 80, 147, 205, 283, 291\].

1.4 Using the RNAi mechanism for therapy

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 \[95\]. 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 \[251\]. miRNAs are important in cell regulation and development \[9, 21, 27, 56, 207\] and they regulate gene expression in humans by translational repression of specific mRNAs or mRNA cleavage and degradation \[10\].

Figure 1.5 depicts the natural miRNA pathway. Polymerase (Pol) II or occasionally Pol III produces the primary transcript, pri-miRNA, that encodes the miRNA \[172\]. Some miRNAs are clustered and transcribed as polycistronic transcripts. The pri-miRNA is processed into the pre-miRNA with 5′-monophosphate and 3′-hydroxyl 2-nucleotide overhang \[40\] by the microprocessor complex that contains the RNase III-like enzyme Drosha and the dsRNA-binding protein DGCR8/Pasha \[75, 108, 120, 168, 171\]. The miRNAs encoded within introns (mirtrons) are processed into pre-miRNAs through a distinct route that uses the splicing machinery as depicted in Figure 1.5 \[50, 250\]. The pre-miRNA is formed in the nucleus and exported to the cytoplasm by Exportin-5 (Exp-5) \[42, 193, 329\]. The RNase III-like endonuclease Dicer subsequently cleaves the base-paired stem approximately 22-basepairs (bp) away from its base, generating a 2-nucleotide overhang at the 3′ end \[333\]. Dicer is associated with the TAR RNA binding protein (TRBP), which is required to recruit Argonaute 2 (Ago2) \[63\]. The Ago2-RNA complex forms the minimal core of the RNA-induced silencing complex (RISC) \[107, 196\]. RISC unwinds the miRNA and loads one RNA strand (guide strand) in the complex, the other strand is degraded (passenger strand) \[118\]. The RNA strand with its 5′ end at the side of the duplex with the lowest thermodynamic stability gets preferentially incorporated into RISC \[203, 294\].

In mammals, post-transcriptional silencing is mainly elicited by translational repression of the targeted mRNA \[10\]. An important determinant is the level of base pairing complementarity between the miRNA and the mRNA target, leading to
1.5. THE BASIC PRINCIPLES OF AN RNAI GENE THERAPY AGAINST HIV-1

mRNA cleavage (perfect complementarity) or translational repression (near-perfect complementarity) \[47, 82, 156, 167, 180\]. RISC typically 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 3' UTR targets and their distance determines the silencing efficiency [254]. Most mammalian miRNAs anneal through imperfect base pairing complementarity with the mRNA to cause translational repression, but at least one case of perfect complementarity and mRNA cleavage is known in humans [328]. Endonucleolytic cleavage of the targeted mRNA occurs opposite of nucleotide position 10 to 11 of the miRNA and the cleaved mRNA is subsequently degraded.

In contrast to natural miRNAs, siRNA with full base pairing complementarity can direct mRNA cleavage with only a single target site that can be located anywhere within the mRNA. Such artificial dsRNA can be produced by several methods. Synthetic mature siRNAs can be transfected into cells [90], but short hairpin RNAs (shRNAs) [50, 228] and artificial miRNAs are expressed intracellularly from a transgene construct [331]. The natural miRNA pathway can be instructed with the man-made inhibitors for therapeutic down regulation of a specific mRNA. This therapeutic approach is relevant for diseases caused by overexpression of a specific mRNA or to specifically target the RNA genomes of invading microbes such as HIV-1.

Instruction of the cellular miRNA pathway with new siRNA specificity is associated with certain risks. One potential problem is direct competition of the artificial siRNAs with the endogenous siRNAs and/or saturation of the miRNA pathway. Since the miRNA pathway is important in the control of cellular gene expression this can have unwanted side effects such as cell death, disturbances in cell differentiation programs or even cancer. Saturation of the miRNA pathway can lead to death when high doses of shRNAs are delivered by an adeno-associated virus (AAV) vector in mice [44, 58, 110, 204, 291, 306]. Another potential problem is targeting of other mRNAs as the miRNAs require only a seed sequence complementarity of 7 to 8 base pairs within the 3' UTR of a given mRNA for function [48]. Such “off-target” effects can be elicited by the passenger and/or guide strand [92, 138, 139]. Yet another problem relates to the induction of an immune response by siRNAs and shRNAs [49, 270], which can be avoided by optimal design of the si/shRNA molecule [199].

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

The main focus of an RNAi-based gene therapy against AIDS would be the durable protection of HIV susceptible cells of the immune system, being the CD4-positive 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 will prevent opportunistic infections and progression towards AIDS. HIV-1 causes a chronic infection and no viral clearance occurs and therefore continuous treatment is required. Repeated delivery of exogenous siRNAs
Figure 1.5: The natural miRNA pathway. For details see text.
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as anti-HIV therapy has been described in a humanized immune system mouse model [162]. This study showed effective virus inhibition and the loss of CD4-positive T cells was prevented. However, it is doubtful if such an approach would be suitable in a patient setting. Since treatment has to be given repetitively in order to obtain the continuous presence of an effective dose of siRNA in HIV-1 susceptible cells that are present in many different body compartments to prevent viral escape. Thus, we strongly prefer the concept of constitutive expression of anti-HIV molecules obtained after 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 to other 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 [217].

Figure 1.6 depicts the gene therapy procedure, including the lentiviral vector production scheme. Several studies indicate that hematopoietic stem cells (HSCs) are not susceptible to HIV-1 infection [313, 334]. HSCs are an excellent candidate for intracellular immunization with the RNAi therapy because they seed the different lineages of immune cells in the blood. The therapeutic lentiviral vector will equip all derived immune cells with the antiviral arsenal. In addition, preferential survival 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 should achieve a durable effect. Clinical trials with hematopoietic stem cells transduced with retroviral delivered anti-HIV-1 ribozymes have previously been performed [8, 212]. These trials demonstrate the feasibility and safety of the proposed stem cell approach. Another option is the treatment of the mature CD4-positive T cell population; in which case repetitive gene therapy should be applied because T cells have only a limited life span [84]. In contrast, the transduced stem cells should continue to generate immune cells of different lineages.
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Figure 1.6: RNAi gene therapy for HIV-1. The HIV-1 infected patient that fails on regular antiretroviral therapy (1) could be offered the RNAi-based gene therapy with a lentiviral vector. The lentiviral vector is produced in 293T cells (2) transfected with the lentiviral vector (e.g. JS1) and a standard set of packaging plasmids (pRSV-rev, pVSV-g and pSYNGP). The lentiviral vector will produce viral genomes and the packaging plasmids will produce the proteins required to assemble new viral particles. pVSV-g produces the vesicular stomatitis virus glycoprotein that is used for virus pseudotyping. Virus particles are collected after 2 or 3 days. The patient will undergo an apheresis for the collection of hematopoietic stem cells after pretreatment with granulocyte colony stimulatory factor (G-CSF) that mobilizes these cells from the bone marrow into the periphery (3). The hematopoietic stem cells will be purified and transduced with the therapeutic lentiviral construct (4). This intracellular immunization with the antiviral shRNA will protect these cells against HIV-1. Transduced cells will be infused back into the patient (5) and the HIV-resistant immune cells will hopefully prevent disease progression towards AIDS (6).
1.6 Scope of this thesis

We studied in detail the means by which HIV-1 can escape from inhibitory shRNAs that are targeting highly conserved regions in the viral genome (Chapter 2). Analysis of the viral population was performed immediately after escape occurred. Subsequent analysis of the observed escape patterns indicated a striking similarity with sequence variation observed in natural HIV-1 isolates. shRNAs can thus be used as a tool to probe the evolutionary sequence space available in the shRNA-targeted region of the HIV-1 genome (Chapter 3). Next, we wondered if there is a difference in early versus late sampling of escape viruses. Late sampling may allow the selection of the most fit and/or most RNAi-resistant variants (Chapter 4). A selection of potent shRNAs was previously made [290] and we now defined which inhibitors yield durable HIV-1 inhibition. In addition, we compared different methods that can be used to validate candidate shRNA inhibitors (Chapter 5). Four shRNAs that elicit durable virus inhibition of more than hundred days were identified.

It is not likely that potent inhibitors can prevent viral escape in a mono-shRNA therapy setting, as a single point mutation can render the virus resistant. Simultaneous expression of multiple potent siRNAs can potentially prevent viral escape. Therefore, we studied different methods of multiplexing antiviral siRNAs against HIV-1 in a single transcript. In Chapter 6 we explore the possibilities of using the naturally occurring polycistronic microRNA cluster present in the MCM7 intron for this purpose. We modulated this construct to express multiple siRNAs against HIV-1 and tested a combinatorial approach with another antiviral RNA molecule (TAR-decoy RNA). In Chapter 7 we explore the possibilities of multiplexed siRNAs expressed consecutively from the U6 polymerase III promoter in a single transcript. In Chapter 8 we propose the use of viral escape analysis as a tool to evaluate the selection pressure imposed by multiple inhibitors in a combinatorial RNAi therapy.

When moving from bench to clinic, the safety and efficacy of an RNAi gene therapy has to be demonstrated. To test the principle of an shRNA stem cell gene therapy we used a novel mouse model with a human immune system. In this model we demonstrated sequence-specific HIV-1 inhibition in the shRNA-expressing T cells that develop from the engrafted lentiviral vector-transduced human stem cells (Chapter 9). Immune cell differentiation did not show differences between treated and control cells. However, a reduction in the number of transduced cells was observed in the shRNA-expressing cells versus the control cells transduced with the empty lentiviral vector. The possible causes of the reduction in GFP-positive cells in the hematopoietic stem cells are discussed in Chapter 10. The current status and future perspectives for moving from bench to clinic with an anti-HIV RNAi gene therapy are summarized and discussed in Chapter 11.