Summary

Over the past 25 years, almost 60 million persons worldwide have been infected with human immunodeficiency virus type 1 (HIV-1) and nearly 25 million have died of the virus-induced acquired immunodeficiency syndrome (AIDS). Currently, a combination of antiretroviral drugs is used to effectively control HIV-1 infection in patients. However, the current drug regimens are expensive, not curative and chronic usage is associated with a significant degree of toxicity for some patients. Drug compliance by the patients is sometimes hampered by the side effects of the drugs. A major threat to the current HIV-1 therapy is the emergence of drug-resistant HIV-1 strains that can spread in the population. Therefore, it is essential that alternative or additional therapeutic approaches against HIV-1 are explored. An attractive and innovative approach is the use of gene therapy to deliver antiviral genes that interfere with viral replication to HIV-1 susceptible cells. Such a gene therapy approach has the potential advantage that the patient will no longer require daily medication. The recent discovery of RNA interference (RNAi) offers a novel method to suppress HIV-1 replication via gene therapy approaches. RNAi is a powerful sequence-specific gene regulation mechanism triggered by double-stranded RNA. The most important effectors of this mechanism are "small interfering RNAs" (siRNAs) and "microRNAs" (miRNAs). These siRNAs and miRNAs are incorporated into the so-called "RNAi-induced silencing complex" (RISC) and guide this protein complex to cleave the complementary mRNA target in the cell. Intracellular expression of short hairpin RNAs (shRNAs) has been developed as a tool to trigger RNAi in a specific manner. These shRNAs are processed into siRNAs by the cellular Dicer protein and are incorporated in RISC to target the homologous transcripts. The use of shRNAs allows researchers to target and inhibit expression of known genes including viral genes. Due to its efficiency and specificity, an RNAi-based gene therapy against HIV-1 may hold great promise as an alternative method to treat HIV-1 infected patients.

Although RNAi against HIV-1 is very potent and specific, studies show that it is difficult to maintain control over HIV-1 because of its high mutation rate. Targeting of a single viral sequence in the HIV-1 RNA genome using RNAi led to the emergence of virus escape variants that acquired mutations within the target sequence or mutations that induce an altered local RNA structure that protects the target from RISC attack. To prevent viral escape, HIV-1 should be targeted simultaneously at multiple viral sequences. In this thesis, we describe the development of novel combinatorial RNAi-based gene therapy approaches against HIV-1. In **chapter 1**, HIV-1, the disease course of HIV-1 infection and progression to AIDS, the current antiretroviral therapy and gene therapy approaches against HIV-1 are introduced. Furthermore, the RNAi mechanism and the strategies to induce RNAi are described.

To facilitate the development of combinatorial RNAi attack against HIV-1, we first designed extended short hairpin RNAs (e-shRNAs) that consist of 2 individual, effective shRNA-units that are stacked on top of each other (**chapter 2**).

We constructed and tested two sets of antiviral e2-shRNAs and examined their silencing activities on luciferase reporters and HIV-1 production. We showed that the siRNA derived from the base of the e2-shRNA is efficiently produced and fully active. However, the top siRNA was only produced when the hairpin stem reached a length of 43 base pairs. Importantly, the properly processed e2-shRNAs can potently inhibit HIV-1 without inducing the non-specific interferon response.

Next, we designed and tested antiviral e-shRNAs that encode 3 or 4 siRNAs (**chapter 3**). We demonstrated that siRNA production and the antiviral effect is optimal for e3-shRNA of 66 base pairs. Further extension of the hairpin stem resulted in an overall reduction of the RNAi activity. The same was observed for long hairpin RNAs (lhRNAs) that target consecutive HIV-1 sequences. Importantly, we showed that HIV-1 replication is potently inhibited in T cells expressing such an e3-shRNA antiviral molecule, whereas viral breakthrough was observed in cells expressing the individual shRNA from the base.

In **chapter 4**, we developed an anti-HIV-1 construct based on a cellular miRNA polycistron. This construct encodes four different siRNAs against HIV-1 from a single transcript. We designed the hairpin RNAs to harbour structural features (mismatches, bulges and thermodynamic stability) that mimic the natural miRNA polycistron as much as possible. We showed that an antiviral miRNA construct can have a greater intrinsic inhibitory activity than a conventional shRNA construct. When combined in a polycistron, the silencing activity is boosted further. Finally, we demonstrated that HIV-1 replication can be efficiently inhibited by simultaneous expression of 4 antiviral siRNAs from the polycistronic miRNA transcript.

We next assessed the ability of antiviral miRNAs and shRNAs to target partially complementary HIV-1 mRNA sequences (**chapter 5**). We tested the inhibitory activities of miRNAs and shRNAs on wild-type HIV-1 and variants that escaped from RNAi-mediated inhibition with one or two point mutations in the target sequence. We showed that miRNAs and shRNAs can significantly inhibit production of HIV-1 variants with mutated target sequences in the open reading frame. A more pronounced knockdown was observed when the target is located in the 3′ UTR of the mRNA. Targeting of multiple imperfect target sites resulted in enhanced knockdown. These data suggest that targeting of imperfect target sites by antiviral miRNAs or shRNAs provides an alternative RNAi approach for inhibition of mutation-prone viruses.

For the delivery of RNAi inducers to HIV-1 susceptible cells, the HIV-1 based lentiviral vector is a good candidate. This is due to the fact that lentiviral vectors are efficient in transducing non-dividing cells, including hematopoietic stem cells, and its ability to stably integrate transgenes into host cell genome. However, the presence of antiviral shRNAs and miRNAs can negatively affect the lentiviral vector titer, which may complicate clinical applications. In **chapter 6**, we analyzed the titer reduction of lentiviral vectors encoding anti-HIV-1 shRNAs and miRNAs. We showed that shRNAs that

target the vector genomic RNA strongly reduce the vector titer, but inhibition of the RNAi pathway via saturation can rescue the titer. For miRNA-vectors, the major cause for titer reduction is promoter interference by the presence of a constitutive promoter. In addition, the presence of miRNAs in the vector RNA genome can cause destruction of the vector genomic RNA due to Drosha processing. Based on these insights we recommend the use of inducible promoters for the expression of miRNAs to prevent titer reduction of these vectors.

In **chapter 7**, a review on lentiviral vector delivery of RNAi effectors against HIV-1 is presented. This review addresses the critical issues regarding lentiviral vector production and discusses the potential for clinical application of RNAi-based gene therapy approaches against HIV-1 using lentiviral vectors.

In **chapter 8**, we review the different strategies to induce combinatorial RNAi for the application against cancer and viral infections. The most straightforward strategy is to express multiple identical or different shRNAs from independent gene expression cassettes. E-shRNA or antiviral polycistronic miRNA constructs, which are presented in this thesis, provide two other attractive methods to induce combinatorial RNAi, which are presented in this thesis. Another approach is to express long hairpin RNAs from which multiple siRNAs could be produced. We address several critical issues including safety and efficacy of combinatorial RNAi gene therapy approaches for a possible clinical application against cancer and viral infections.