Silencing of HIV-1 co-factors
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Chapter 1
General introduction
HIV-1

The first cases of the Acquired Immuno Deficiency Syndrome (AIDS) were reported in 1981, and two years later the causative agent was isolated (30;125). The virus appeared to be a member of the lentiviridae, a subfamily of the Retroviridae. Most viruses from this family are known to cause chronic diseases of the central nervous system and the immune system. The virus was eventually named Human Immunodeficiency Virus type 1 or HIV-1.

It is estimated that globally 33.3 million people were living with HIV-1 at the end of 2009 and 2.6 million people became newly infected that year. Most of the people infected with HIV-1 live in Sub-Saharan Africa and Southeast Asia (www.unaids.org).

HIV-1 molecular biology

The HIV-1 genome (Figure 1.1) is 9.8 kb in length and encodes nine viral genes; gag, pol, env, tat, rev, nef, vpu, vpr, and vif (284;365). The gag, pol and env genes are typically found in all retroviruses. The gag gene encodes four structural proteins; the p24 capsid (CA), the p17 matrix (MA), the p7 nucleocapsid (NC) and the p6 protein. Pol encodes for a polyprotein that is post-translationally cleaved into three enzymes; reverse transcriptase (RT), integrase (IN) and protease (PR), all three indispensable for the viral replication cycle.

The env gene encodes a protein that is cleaved into two subunits; the glycoprotein 120 and glycoprotein 41, both subunits of the trimeric Envelope protein and important for recognition of receptors on the host cell. The tat and rev genes encode for the regulatory proteins Tat and Rev, which are essential for viral replication; Tat activates viral transcription and Rev is necessary for nuclear export of viral RNAs out of the nucleus (256;292). The HIV-1 genome encodes four accessory proteins: Nef, Vpu, Vpr and Vif. These proteins are not essential for viral replication in many in vitro systems, but are needed for viral replication, viral spread and pathogenesis in vivo (16;108). Nef has been implicated in several biological processes; e.g. down regulation of CD4 and Major Histocompatibility Complex molecules, enhancement of virion infectivity and stimulation of viral replication in primary T cells (119). Vpu contributes to CD4 downregulation by mediating degradation of newly synthesized CD4 molecules in the endoplasmatic reticulum. Vpu also enhances virus particle release by antagonizing the host restriction factor tetherin (99). Vpr interacts with many cellular proteins and has been implicated in several different functions, such as improving the fidelity of reverse transcription, suppression of immune activation and induction of G2 arrest and apoptosis (291). Vif protects HIV-1 from the host restriction factor APOBEC (134).

The protein-coding regions in the genome are flanked by non-coding domains; the Long-Term Repeats (LTRs), which are subdivided into the U3 (Unique 3’), R (Repeat) and U5 (Unique 5’) domains (127). These DNA domains encode the viral promoter and several RNA signals that are important for viral replication. For instance, the TAR (trans-activation response) RNA hairpin binds the Tat protein and is essential for Tat-mediated activation of transcription. Several transcription factors bind to the LTR, such as Sp1, NF-κB, NF-AT and USF (271;281).
Chapter 1

HIV-1 replication cycle

Replication of HIV-1 starts when the virus encounters a host cell expressing the CD4 receptor and the CXCR4 or CCR5 co-receptor (Figure 1.2). These receptors are present on certain immune cells, especially CD4+ T lymphocytes, macrophages and dendritic cells. The Envelope protein on the outside of the virus particle binds to the CD4 receptor on the target cell, which leads to conformational changes in the Envelope protein. The second step of the virus entry process is binding to the co-receptor, in primary infections the CCR5 chemokine receptor, and subsequent fusion of the viral membrane with the target cell membrane. The contents of the virion are released into the host cell. The viral RNA genome is reverse transcribed into DNA by the viral Reverse Transcriptase and the capsid is partially disassembled. The capsid, with the viral genome is transported to the nucleus and the viral genomic DNA is subsequently integrated into the host genome by Integrase. The integrated provirus acts as a template for the production of spliced viral mRNAs and full-length RNA genomes. Viral mRNAs are translocated to the cytoplasm and translated into viral proteins. The viral proteins are, together with two copies of progeny RNA genomes, assembled in new virions at the plasma membrane, and released from the cell by budding. After maturation, the new virions are able to infect new host cells. The viral replication steps from entry to integration are referred to as “early” replication steps, while the stages from transcription to budding of new virions are referred to as “late” replication steps (121).
**Treatment of HIV-1 infection and AIDS**

AIDS has become a chronic disease in the Western world due to the introduction of combined anti-retroviral therapy (cART), although people living with HIV-1 still have a lower life expectancy than uninfected individuals (414). In the early days of the disease, only a single class of drugs was available and drug resistance emerged quickly due to the error-prone Reverse Transcriptase (195). cART uses a combination of drugs and was introduced in 1996, which led to the first decrease in annual AIDS deaths in the USA since the beginning of the epidemic. cART is advised to patients with a CD4 count below 350 cells/mm3, while other drugs (e.g. antibiotics) can be added to the regimen after the onset of AIDS to treat opportunistic diseases. Nowadays the following antiviral drug
classes are available: NRTIs and NNRTIs (nucleoside and non-nucleoside Reverse Transcriptase inhibitors), Protease inhibitors (PI), Integrase inhibitors and entry inhibitors (CCR5 antagonist and fusion inhibitors) (see also Figure 1.2). The most common cART drug combination consists of two NRTIs with a single PI or NNRTI. However, several drawbacks of cART have been recognized. The drugs have been associated with side-effects, from mild ones as nausea and fatigue to severe side-effects such as organ failure (10). Patients have to live with a daily drug regimen and sub-optimal drug levels in patients that do not strictly adhere to the medication can lead to the emergence of drug-resistant viral strains (315). The life-long medication is also expensive. Perhaps most importantly, none of the drugs is able to eradicate the virus from the body. As the development of a vaccine is still unsuccessful, despite intense efforts, there continues to be a real need for development of new therapeutics.

**RNA interference and its use against HIV-1**

RNA interference or RNAi is an evolutionary conserved mechanism in eukaryotes that leads to sequence-specific knockdown of gene expression (116). The hallmark molecule of this pathway is double stranded RNA (dsRNA), which can originate from different sources, such as the dsRNA intermediate in the replication of viruses, or dsRNA encoded by the genome as microRNAs (miRNAs). MiRNAs are important in cell differentiation and development and regulate gene expression by either translational repression of specific mRNAs or mRNA cleavage. For more information on the natural RNAi pathway, see Chapter 2.

![Figure 1.3. RNAi pathway](image)

ShRNAs can be produced from plasmid DNA or vectors in the nucleus and are translocated into the cytoplasm via Exportin-5. Dicer cleaves the shRNA into a siRNA. Alternatively, siRNAs can be transfected into the cells. The siRNA
General introduction

The natural RNAi pathway can be instructed by man-made dsRNA molecules designed to target the mRNA of interest. This can be done by transfecting synthetic small interfering (siRNAs) into cells (105), by intracellular expression of short hairpin RNAs (shRNAs) or artificial miRNAs from DNA constructs (60;399). These artificial RNAi inducers are loaded into the RNAi pathway and can be used for therapeutic downregulation of a target gene (Figure 1.3). There are certain risks associated with the use of RNAi. Overexpression of siRNAs, either by transfection or by expression from a vector, may lead to saturation of the cellular RNAi pathway, which can lead to cell death, disturbance in cell development and even cancer (140). Another risk is the so-called “off-target” effect. As a miRNA only need 7 or 8 base pairs of sequence complementarity, there is always the possibility that not only the intended mRNA is targeted, but unrelated mRNAs as well (306). When shRNAs or siRNAs are suboptimally designed, they can also induce immune responses (58;324). However, all these problems can be circumvented or checked for, which makes the use of RNAi an appealing therapeutic possibility in the treatment of a wide variety of diseases (20;46;267;314).

In HIV-1 therapy, the RNAi treatment should be delivered cells that are susceptible to virus infection, being CD4+ T lymphocytes, macrophages and dendritic cells. Transient delivery of siRNAs has been tried and proven successful in inhibition of HIV-1 replication in a mouse model; but this approach will not be suitable for patients (188). The ultimate goal is to treat HIV-1 susceptible cells to allow the constitutive expression of antiviral shRNAs. This can be achieved by transduction of these cells with a lentiviral vector. Lentiviral vectors are derived from HIV-1 and can infect cells to integrate the therapeutic RNAi cargo into the cellular genome, but are unable to replicate (100). Lentiviral vectors can transduce many cell types, both dividing and quiescent, making these vectors ideal candidates for a gene therapy against HIV-1 (254). The target cells could be hemapoietic stem cells (HSC), which give rise to several lineages of immune cells, including HIV-1 susceptible cells such as CD4+ T lymphocytes. As HSC are long-living cells that continue to generate new immune cells, in theory a single gene therapy treatment should suffice for a life-long generation of protected cells.

In principle one would like to target early replication steps of HIV-1, and prevent proviral integration into the host genome. However, it appears that the incoming viral RNA genome is not a target for RNAi, as it is highly structured, coated with viral and cellular proteins and hidden in the viral capsid and thus inaccessible for the RNAi machinery (381). Late in infection, the newly made HIV-1 mRNAs will be targets for RNAi attack, and several shRNAs designed against conserved viral sequences were tested in vitro, of which several inhibited HIV-1 production and replication substantially. Stable knockdown cell lines expressing individual shRNAs were generated and infected with HIV-1. No replication of HIV-1 was detected for several weeks or even months, but HIV-1 eventually started replicating in these cultures (83). When the replicating virus was analyzed and sequenced, mutations in or close to the siRNA-target sequence were found. Mutations in the mRNA target abolish the perfect sequence complementarity needed for shRNA-mediated gene knockdown (83). A mutation found close to the siRNA-target site did not change the sequence complementarity, but was found to alter the secondary structure of the HIV-1
mRNA, making the target less accessible for the RNAi machinery (380). Both types of mutations made HIV-1 resistant against RNAi attack. Thus, although RNAi against HIV-1 mRNAs can be very effective, solutions are needed to prevent the development of resistant virus variants. Similar to current cART therapy more than one region in the HIV-1 genome can be targeted by the use of multiple shRNA inhibitors, and this was shown to be effective and no escape viruses could be selected (364). An alternative method to prevent viral escape would be to target the cellular proteins that assist HIV-1 to complete its replication cycle. More in-depth information of RNAi strategies against viral and cellular targets is provided in Chapter 2.

HIV-1 encodes only 15 proteins and needs many cellular proteins as co-factors to complete its replication cycle. RNAi has played a major role in the identification of these cellular co-factors. Three genome-wide RNAi screens have revealed many candidates, however, the overlap between these three studies was small and only three co-factors were identified in all three studies (53;62;181;406). This shows the importance for confirmation of candidate cellular co-factors by additional experimentation. The overlap in the pathways involved was more significant. Targeting of cellular co-factors has several advantages; it is possible to prevent proviral integration by targeting co-factors necessary for the early replication steps, e.g. viral entry. In theory one would expect that the chance of viral escape is much reduced as HIV-1 has evolved to very specific cellular co-factors, which makes it less likely that it can switch to the use of another related cellular co-factor. The main is that targeting of cellular co-factors could have adverse effects on cell growth and/or function, thus leading to side effects.

Scope of this thesis

In Chapter 2 a detailed overview is presented on how and why a gene therapy against HIV-1 can serve as a new therapeutic option. The focus is on the rationale of using RNAi and whether to target the virus or cellular co-factors and ongoing clinical research is reviewed. The main body of this thesis concerns the use of RNAi against cellular co-factors as antiviral approach that may prevent the selection of RNAi-resistant escape viruses. Stable knockdown cells with shRNAs against 30 different cellular co-factors were generated and challenged with HIV-1 in Chapter 3. In three cases we could inhibit HIV-1 replication up to two months and no viral escape was apparent.

Adverse effects on cell physiology due to RNAi attack on cellular co-factors are a major concern. To accurately measure even small effects on cell growth, either due to specific knockdown of the target or unknown off-target effects, we developed a new and sensitive cell proliferation assay in Chapter 4. In this competitive cell growth or CCG assay a mixture of transduced GFP-positive cells and untransduced GFP-negative cells is cultured over time. If transduced cells have a lower proliferation rate, this can be easily scored by FACS analysis as a gradual decrease in the percentage of GFP-positive cells. We show that the CCG assay is more sensitive than other well-established cell proliferation assays.

In Chapter 3 we obtained long-term inhibition of HIV-1 replication and one of the top candidates from this screen was an autophagy factor. Autophagy is a cellular pathway and its basic function is to recycle cellular components. Autophagy has also been implicated in HIV-1 replication and therefore we tested RNAi-mediated knockdown of additional autophagy factors. In Chapter 5 knockdown cells expressing shRNAs against different autophagy factors were generated and HIV-1 replication was inhibited in several of these.
When simultaneously knocking down two autophagy factors, we obtained increased virus inhibition without enhanced cytotoxicity compared to the individual knockdown cells. In Chapter 6 we study Cyclophilin A and B, cellular proteins that both have isomerase function. Cyclophilin A is known to support HIV-1 replication, but we have identified Cyclophilin B as a HIV-1 restriction factor. Thus, proteins with a similar enzymatic function can acts as cellular co-factor or as restriction factor. The DDX3 helicase supports HIV-1 replication through interaction with Rev and facilitating the nuclear export of viral transcripts. In Chapter 7 we demonstrate that DDX3 is also involved in Tat-mediated trans-activation of the HIV-1 LTR promoter, making DDX3 an interesting therapeutic candidate with a dual co-factor function. By targeting a single cellular co-factor, one could block two steps in the HIV-1 replication cycle. The general discussion in Chapter 8 provides a short review of RNAi against cellular co-factors in the context of other pathogens, such as influenza A virus and hepatitis C virus and the intracellular bacterium *Mycobacterium tuberculosis*. 