Silencing of HIV-1 co-factors
Eekels, J.J.M.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 8
General discussion
Lessons from RNAi silencing of HIV-1

RNA interference (RNAi) is an evolutionary conserved mechanism induced by double-stranded RNA (dsRNA) that triggers sequence-specific gene silencing at the post-transcriptional level. The dsRNA-inducer molecule consists of 19-23 nucleotides with one strand complementary to the target mRNA (116). Intracellularly expressed short hairpin RNAs (shRNAs) as well as transfected small interfering RNAs (siRNAs) have been successfully used against target sequences in the HIV-1 RNA genome (76;345). However, resistance mutations can be selected when a single viral RNA sequence is targeted in a monotherapy setting. Furthermore, since the RNA genome from infecting virion particles is not a target for RNAi attack, the virus can only be inhibited at the level of the de novo synthesized RNA transcripts, thus after integration of the provirus into the host cell genome. Therefore, targeting of the cellular co-factors that are important for virus replication may provide several advantages. First of all, it has been hypothesized that it is more difficult for the virus to gain resistance against such inhibitors, unless HIV-1 can evolve to use a related co-factor (276). Second, targeting of cellular co-factors that are important in early replication steps can prevent the integration of HIV-1 DNA into the cellular genome and thus the establishment of the stable provirus reservoir. The best example is the HIV-1 CCR5 co-receptor. Persons who are homozygous for a 32-bp deletion in the CCR5 gene (CCR5Δ32) are healthy and resistant to HIV-1 infection as in most cases CCR5-tropic HIV-1 variants are transmitted (89;156). CCR5 facilitates the first step of HIV-1 replication; entry of the virus into the target cell. Targeting of CCR5 is therefore often presented as the perfect candidate in a therapy based on targeting of cellular co-factors. However, HIV-1 may switch to use the CXCR4 co-receptor. Third, it is possible that different viral pathogens use the same cellular co-factor, which could thus be a broad-spectrum therapeutic target.

In this thesis we tested whether RNAi against cellular co-factors can be used to inhibit HIV-1 replication. Therefore we choose 30 candidate co-factors based on a literature survey and generated stable knockdown T cell lines. This enabled the long-term analysis of the effects of host protein silencing on viral replication and, perhaps equally important, on cell viability. In any setting where long-term culturing of cells is involved, a small delay in cell proliferation can eventually have a major impact on the experimental outcome. A major impact on cell proliferation can easily be scored by visual inspection of the cell cultures and by the practical observation that some cultures need to be passaged less frequently than others. However, subtle differences in cell growth are not easily detected. Several methods exist to determine cell viability and proliferation, but we demonstrated that these assays are usually not as sensitive as one would like. To detect smaller deficiencies in cell proliferation, we designed the competitive cell growth or CCG assay, which exhibits a higher sensitivity than currently available assays.

With the CCG assay we were able to determine the effect of co-factor silencing on cell growth. In some cases fairly small defects were measured (1-5% reduced cell growth), which is a difference that easily will go unnoticed during culturing. For other co-factors we measured a higher impact on cell growth. One can ask whether this poses a problem in a gene therapy setting. The answer obviously depends on the cell types that are targeted in the RNAi therapy and their proliferation capacity. Another critical issue is whether the cell function is altered by co-factor silencing, which requires additional experimentation.
However, when cell growth is affected by a treatment that makes the cells non-
susceptible for HIV-1 replication, one could hypothesize that the therapeutic value
outweighs the negative effect on cell growth.

To obtain a prolonged inhibition of virus replication as shown in Chapter 3, a high amount
of lentiviral vector input was needed and eventually only breakthrough viruses and no
true viral escape were observed. This can be explained by the fact that no complete
knockdown was achieved for any of the cellular co-factors tested in this thesis. Although
this may also be the case for HIV-1 RNA knockdown, the situation changes when the virus
replication cycle is taken in account. In case of targeting an early viral mRNA, the cascade
of viral gene expression may actually result in an enhanced reduction of late viral mRNAs
such that viral replication is seriously affected. One can of course boost the antiviral effect
when targeting cellular co-factors by the use of additional shRNAs against the same co-
factor (intensified RNAi). This approach was used for the cellular co-factor LEDGF/p75, for
which a nearly complete knockdown is needed to score an effect on HIV-1 replication
(223). The antiviral effect can also be boosted by combining shRNAs against multiple
cellular co-factors. We show in Chapter 5 that simultaneous targeting of 2 autophagy
factors increases the inhibitory effect on HIV-1 replication. However, it appears that the
knockdown efficiency required for HIV-1 inhibition differs significantly per co-factor. For
the DDX3 helicase a modest knockdown of 55% at the mRNA level was sufficient to score
inhibition of virus replication.

Targeting cellular co-factors of pathogenic viruses

In this thesis we describe the use of RNAi against cellular co-factors of HIV-1 replication.
However, this approach can be used for the therapeutic silencing of other viruses as well.
RNAi screens have recently been performed for several pathogens to identify candidate
co-factors that are critically involved in the pathogen replication cycle. We will present
those RNAi screens and antiviral RNAi approaches and will start with influenza A virus, for
which several RNAi screens have been reported.

Influenza A virus

Influenza A virus is a member of the genus Orthomyxoviridae and is an enveloped,
negative-stranded RNA virus. It is well known for causing the flu in humans and can infect
e.g. birds and pigs as well. Influenza viruses cause annual epidemics and sporadic
pandemics, with the potential of severe pathogenicity in humans. The last pandemic in
2009 was caused by the swine-derived H1N1 virus and caused around 60 million infections
worldwide, although most cases reported only relatively mild symptoms. Several drugs are
available for prophylaxis and treatment: oseltamivir and zanamivir (both inhibitors of the
viral neuraminidase protein) and amantidine and rimantadine (blocking uncoating of the
virus via inhibition of the viral M2 protein). Most human influenza variants are resistant
against the latter class of drugs (59) and several strains are resistant against oseltamivir
(351).

Influenza encodes only 10 proteins and therefore requires the cellular machinery and
many of its proteins as co-factors in the replication cycle. Although the function of the
viral proteins has been studied extensively, many of the important co-factors remained
elusive. Several genome-wide studies have been conducted to identify candidate co-
factors and for influenza no less than 6 RNAi studies were performed. The first study by Hao et al was done in Drosophila cells as mammalian cell systems were not yet available (149). Although RNAi-based screening works well in insect cells, the virus had to be modified to allow replication in Drosophila cells. Over 100 Drosophila genes were identified as being involved in influenza virus replication. Next, three genome-wide RNAi studies were performed in mammalian systems. Brass et al used human osteosarcoma cells (55), while König et al and Karlas et al used the human lung cell line A549 (172;179). The studies of Brass and König were designed to identify cellular co-factors involved in the early to mid-replication cycle, while the experimental set-up of the Karlas study assessed the complete replication cycle up to budding of new virions. Many possible co-factors were described; Brass listed 133, König 295 and Karlas 287 candidates. Two studies used quite different approaches to identify co-factors of virus replication. Shapira et al combined yeast-2-hybrid screening with gene expression profiling and validated the resulting 1745 hits with siRNA-mediated silencing in virus replication assays. Subsequently the effect of siRNA-mediated knockdown on the interferon-β response was measured in experiments using transfection of viral RNA and infection with a ΔNS1 virus (317). This last virus does not express the NS1 protein that would normally block the interferon response. These validation experiments produced a shortlist of 616 genes. A new technique was employed by Sui et al called “random homozygous gene perturbation” or RHGP (208). This silencing strategy uses antisense RNA generated from an integrated genetic element with a promoter. This genetic element integrates via lentiviral mediated transduction at a single allelic site in the genome, either in sense or antisense orientation. In sense orientation, insertion of a promoter element upstream of the gene will lead to overexpression, such that gain-of-function experiments can be performed. In antisense orientation, the antisense transcript can silence the other allele in trans, such that knockdown studies can be performed. The major advantage of this system is that no prior knowledge or annotation is needed about candidate genes. After screening for the desired phenotype, the cell clone can be analyzed to identify the affected gene. This screen revealed 110 human genes that render host cells resistant to influenza infection (331).

It may come as a surprise that there is hardly any overlap between these 6 studies. Pairwise comparisons revealed between 0 to 32 common co-factor candidates. The largest overlap of 32 hits concerned the similar König and Karlas analyses that employed siRNA-mediated silencing in the same cell line. A mere 128 genes were found in common in at least 2 studies. The overlap in common cellular pathways is much higher, e.g. eukaryotic translation initiation, processing of capped pre-mRNAs, and Golgi-to-ER retrograde transport were among the overrepresented pathways (373).

Many studies have tested the use of RNAi therapeutics against the influenza A RNA targets. Several siRNAs against conserved viral targets such as the mRNAs encoding the NP, PA, PB1, PB2, M and NS proteins have been tested. Targeting the HA and NA sequence was also attempted, but a single siRNA could only silence a few viral strains due to sequence variability (130).

**Hepatitis B virus**

Globally some 390 million persons carry hepatitis B virus (HBV) and chronic infection leads to an increased risk for developing cirrhosis and hepatocellular carcinoma (HCC) (21). Between 25 and 40% of individuals who are chronically infected with HBV will develop one
of these complications. Clinical treatment of HBV consists of interferon alpha (IFNα) and nucleoside/nucleotide analogs, which are only partially effective. Prolonged treatment is necessary and often complicated by the emergence of resistant virus variants, and thus new therapy options are needed. As for HIV-1, RNAi against viral targets has been developed and promising results have been obtained by targeting the polymerase gene, including the variants thereof with resistance mutations against the antiviral drug lamivudine (242).

Several host proteins and cellular pathways have been described as being essential for HBV replication, e.g. heterogenous ribonucleoprotein K, heat shock protein 70, the sphingolipid biosynthesis pathway and the DNA damage signaling pathway (257;341;371;403). Although RNAi has been used against these host factors as a research tool to test the impact on virus replication, no therapeutic approach based on RNAi against host proteins has been described yet.

**Hepatitis C virus**

Hepatitis C virus (HCV) is frequently detected in HIV-1 infected individuals, with estimates of 30% double infections among the HIV-1 infected individuals (320). Most acute infections are asymptomatic or cause only mild symptoms. However, similar to HBV infection, the virus can cause a persistent infection that eventually may cause cirrhosis and HCC. Treatment options include a combination of pegylated INFα and ribavirin, but treatment responses range from 45 to 80%. RNAi against viral targets leads to inhibition of viral replication, with prime targets in the 5’-non-translated region (5’NTR) and non-structural genes such as the one encoding the polymerase (186;338). Not surprisingly, HCV can rather quickly develop resistance against RNAi targeting the viral NS5B gene, although the resistant viruses remained sensitive to siRNAs that target other viral sequences (382).

RNAi has been used as a research tool to identify cellular co-factors for HCV replication in two genome-wide siRNA screens (210;336)and three smaller siRNA screens against more specific sets of candidate co-factors (282;283;333). Again, very little overlap in the genome-wide siRNA screens was apparent; none of the 9 hits identified by Li et al were scored by Tai et al, while similar experimental settings were used. Only a single co-factor from a genome-wide screen was also scored in the smaller screen performed by Randall et al (75). Interestingly, DDX3 represents the strongest hit in one of the small siRNA screens, and other studies showed that DDX3-knockdown reduces viral replication (23). It has been suggested that DDX3 interacts with the HCV core protein (265), although this has become the focus of discussion (17). As shown in chapter 7, DDX3 has two functions in HIV-1 replication and stable knockdown did only moderately affect cell proliferation. This makes DDX3 an attractive broad spectrum therapeutic target as it plays important roles in the replication cycle of at least two human pathogenic viruses that frequently cause co-infections.

RNAi against cellular co-factors has been described in therapeutic settings; siRNAs and adenovirus delivered siRNAs against the La autoantigen, polypyrimidine tract binding protein and VAMP-associated protein of 33 kDA were tested (96;401). Another class of proteins that is important for HCV replication concerns the Cyclophilins, and siRNAs against several proteins of this family reduced viral replication (253). Currently, analogues of cyclosporine A such as Debio-025 are being tested for treatment of HCV infection (118). Worth mentioning is that HCV is unique in that it has another special cellular co-factor, the
cellular microRNA miR-122 (166). This miRNA binds to two sites in the 5’NTR of HCV RNA and upregulates viral replication, in part by stimulating HCV translation. A candidate drug in the form of an antisense molecule has been tested in chimpanzees, and a significant reduction in viral load and HCV-induced liver pathology was observed (107). However, viral resistance against the miR-122 antagonist could occur in cell culture by introducing mutations in the target site of miR-122 (212). Long-term tests with miRNA antagonist should reveal whether drug-resistant HCV variants will be selected.

**Herpes simplex virus type 2**

Herpes simplex virus type 2 (HSV-2) causes genital herpes and HSV-2 infection can increase the risk for HIV-1 transmission by disruption of the mucosal membranes in the genital tract. A microbicide against HSV-2 could thus help in the prevention of sexually transmitted diseases (STDs). Intravaginal administration of siRNAs targeting two HSV-2 genes (UL27 and UL29) complexed in a transfection lipid transiently protected mice from HSV-2 infection (124). However, the transfection lipid itself enhanced viral infection, and the authors therefore switched to cholesterol-conjugated siRNAs. In this second study, the viral UL29 gene and the cellular Nectin-1 receptor were targeted with siRNAs. Intravaginal protection against HSV-2 infection was observed for up to a week when mice were treated with these 2 siRNAs (384). This case shows that transient RNAi against a cellular co-factor can be used as a prevention measure against viral infections. A particularly useful property in STD prevention is the fact that this RNAi-based microbicide does not have to be applied immediately prior to sexual intercourse.

**Using RNAi against bacterial infections**

Not only viruses are intracellular pathogens, some bacteria replicate inside host cells as well. Although most of these bacteria do not rely on host proteins for their life cycle as much as viruses do, bacteria do modulate several cellular pathways to make the host cell more permissive for bacterial propagation. Many RNAi studies have been performed to identify cellular co-factors important for infection with Listeria monocytogenes, *Mycobacterium fortuitum* and *tuberculosis*, *Legionella pneumophila* and *Chlamydia spp* (6;74;92;97;272). We will focus here on research performed on *M. tuberculosis* as tuberculosis (TB) is the leading cause of death among HIV-1 infected persons in Africa. *M. tuberculosis* is an obligate human pathogen that primarily targets macrophages, where the bacterium resides in early and late phagosomal compartments. After infection, mostly via inhalation of contaminated aerosols, the bacteria are internalized by alveolar macrophages via phagocytosis (24). Normally, activated macrophages will transfer the phagocytosed bacteria to the lysosomes in order to degrade the pathogen. However, some bacteria escape from lysosomal delivery and survive within the macrophage. Only 10% of infected individuals eventually develop tuberculosis as healthy people can keep *M. tuberculosis* in check. But the combination of HIV-1 and *M. tuberculosis* infection forms a deadly combination, leading to a higher mortality rate than a single infection with either TB or HIV-1. This is further complicated by the fact that many *M. tuberculosis* strains are resistant against the first line antibiotics rifampin and isoniazid and annually 440,000 cases are reported to be multi-drug resistant (MDR) (415). Another challenge in the treatment of co-infections concerns the possible interactions between antiretrovirals and TB drug
regimens. For example, the anti-TB drug rifampin can lead to subtherapeutic levels of HIV-1 protease inhibitors, and thus trigger virus escape and therapy failure (190). The Bacillus Calmette-Guérin vaccine is available for TB, but conflicting studies have shown an efficacy of 0 to 80% (77;290). A new therapeutic approach could include the targeting of host factors.

*M. tuberculosis* has evolved to modulate several host mechanisms in order to survive in the hostile cellular environment. RNAi gene knockdown has played a significant role in elucidating the cellular co-factors that are involved. Two studies have been performed in Drosophila cells with Mycobacterium strains that replicate in insect cells; a genome-wide siRNA screen with *M. fortuitum* (272) and a 1000 target gene RNAi study with *M. marinum* (183). Another genome-wide study was performed in a human macrophage-like cell line with a virulent *M. tuberculosis* strain (187), this study used siRNA pools against more than 1100 genes with antibacterial activity in the initial screen, and subsequent screens followed by individual siRNA validation resulted in 275 candidate co-factors. Interestingly, when siRNAs against the 275 candidate co-factors were tested for inhibition of other virulent and MDR *M. tuberculosis* strains, only 74 showed robust activity against all 8 strains tested. Half of these targets (44 out of 74) are involved in negative regulation of the autophagy mechanism, which may make sense as autophagy is the cellular defense mechanism against intracellular pathogens. Treatment with autophagy-inducing drugs has therefore been proposed as a novel treatment option for MDR *M. tuberculosis* (259). In this thesis we have shown that silencing of individual protein components of the autophagy pathway presents a therapeutic option in the treatment of HIV-1. Thus, extreme caution is warranted to avoid the development of an antiviral strategy (autophagy inhibition) that will boost *M. tuberculosis* or an antibacterial strategy (autophagy induction) that may facilitate HIV-1 replication.

**Concluding remarks**

The examples mentioned above demonstrate the usefulness of RNAi as a research tool to identify candidate co-factors in the replication of viruses, intracellular bacteria and other pathogens. Although not discussed here, these techniques have also been used in other research fields such as cancer and metabolic diseases to elucidate host proteins that are important for e.g. tumor growth or insulin resistance (153;277;319). When more than a single study was performed, the overlap in the identified co-factors was always reasonably small, even when the same cells and virus strains were used. This can largely be attributed to the manner in which such large data sets are analyzed, such as the definition of what is a “hit”. It is clear that the results of such large screens need to be carefully validated. The use of stable knockdown cells instead of short-term gene suppression with siRNAs is important in this respect. On a positive note, several pathogens turn out to use the same cellular pathways or even the same protein co-factors, and this opens the possibility of developing a broad spectrum therapy against multiple pathogens.

For clinical application many hurdles need to be taken, such as the targeted delivery of the RNAi inducer molecule. Several possibilities have been presented in literature, from nanoparticle delivery of siRNAs for transient therapies to the transduction of progenitor target cells with a viral vector with an inducible promoter for the expression of an antiviral shRNA. Several RNAi-based therapies are currently being tested in clinical trials (86;168).