UNIVERSITÀ
DEGLI STUDI
DI PADOVA

UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI MEDICINA MOLECOLARE

SCUOLA DI DOTTORATO DI RICERCA IN BIOMEDICINA
CICLO XXVI

DEVELOPMENT OF LENTIVIRAL VECTORS EXPRESSING MULTIPLE SMALL INTERFERING RNAs AS A GENE THERAPY APPROACH AGAINST HIV-1 INFECTION

DIRETTORE DELLA SCUOLA: CH.MO PROF. RICCARDO MANGANELLI

SUPERVISORE: CH.MA PROF.SSA MARIA CRISTINA PAROLIN

DOTTORANDA: FRANCESCA SPANEVELLO

INDEX

1. SUMMARY

The advent of combinatorial antiretroviral therapy proved to be highly effective in controlling HIV-1 disease progression, transforming AIDS from a deadly to a chronic condition. However, major current issues are systemic drug toxicity, generation of drug resistant viral mutants and persistence of a latent viral reservoir. In addition, drug-based regimens require daily intake and many patients cannot maintain the high level of adherence necessary for viral control. Given the limitations of the current therapeutic approaches and the absence of any effective vaccination strategy against HIV-1 infection, there is a pressing need to develop a curative treatment. The first reported cure of HIV-1 infection was provided by the apparent eradication of the virus in a patient transplanted with hematopoietic stem cells (HSCs) from a donor lacking the functional CCR5 co-receptor, which is used by most HIV-1 strains to enter target cells. However, while an important proof-of-principle, few individuals could benefit from such procedure due to toxicities of allogeneic rejection and limitations of finding compatible CCR5-negative donors. Therefore, gene therapy approaches aimed to modify autologous HSCs in order to render them resistant to HIV-1 infection have emerged as a promising direction. If successfully engrafted, these cells would offer continuous, long-term production of virus-resistant immune cells. To this end, several anti-HIV-1 genes have been developed and tested both in preclinical and clinical settings and, among these, RNA interference (RNAi)-based approaches represent one of the most powerful tools.

Starting from these considerations, this study was aimed to develop lentiviral vectors expressing anti-HIV-1 RNAi triggers and to test their efficacy in relevant human primary cells for HIV-1 infection, including CD4+ T lymphocytes and macrophages. The final goal of the research project is to use the most effective and safe vector(s) to genetically modify HSCs harvested from AIDS-related lymphoma (ARL) patients, that offer a unique opportunity to evaluate anti-HIV-1 gene therapy strategies in an ethically acceptable clinical setting, as they often undergo HSC transplantation.

To account for HIV-1 variability, we selected multiple RNAi triggers, including a short hairpin RNA (shRNA), generating one single small interfering RNA

(siRNA) against either the CCR5 cellular gene (shCCR5) or the *vif* viral gene (shvif), and a long hairpin RNA, giving rise to two different siRNAs against the viral *tat* and *rev* overlapping first exons (lhtat/rev). Overall these molecules inhibit different steps of the HIV-1 life cycle, including entry into target cells (shCCR5), gene expression (lhtat/rev) and infectivity of the newly produced particles (shvif).

In the first part of the work, we constructed vectors expressing the shCCR5, the shvif or the lhtat/rev as a single transcriptional unit under the control of different human polymerase III promoters (i.e. U6, 7SK or H1). These vectors allowed us to investigate promoter influence on the siRNA silencing activity, finding out the best combination of promoter-RNAi trigger for the development of a combinatorial antiviral approach. As a next step, we obtained combinatorial vectors simultaneously expressing the above described siRNAs, as independent transcriptional units within the same vector backbone. To optimize vector design, a number of different vectors were developed by using either the same or distinct promoters driving the expression of each RNAi trigger. Moreover, the transcriptional units were cloned in different position with respect to each other within the vector framework. Considering that the use of multiple highly active RNA polymerase III promoters can potentially saturate the endogenous microRNA biogenesis pathway, we also explored an alternative combinatorial strategy, based on the use of an extended shRNA (e-shRNA). This molecule simultaneously expresses the three siRNAs targeting the CCR5, the *vif* and the *tat*/*rev* transcripts, under the control of the U6, the 7SK or the H1 promoter. When comparing the antiviral activity of all the different combinatorial platforms, we could identify two vectors (i.e. U6shCCR5-7SKshvif-H1lhtat/rev and H1e-shRNA) conferring highly potent protection against HIV-1 infection in both cell lines and human primary cells, in the absence of cytotoxicity.

Overall, our findings highlighted some important strengths and pitfalls of different approaches used for multiple siRNAs delivery, providing valuable insights for the design and application of reliable combinatorial RNAi to counteract HIV-1 replication. In addition, this study contributed to the identification of new anti-HIV-1 combinatorial platforms that, once shown to be effective and safe *in vivo*, may be next in line for clinical testing.

2. SOMMARIO

Nonostante l'impiego di associazioni di farmaci nel corso dell'infezione da HIV-1 determini una riduzione della carica virale e ritardi la progressione della sindrome da immunodeficienza acquisita (AIDS), la tossicità dei farmaci, la comparsa di virus resistenti e la persistenza del virus in specifici compartimenti anatomici e cellulari rappresentano una sfida per il controllo a lungo termine dell'infezione. In tale contesto si inserisce il crescente interesse della comunità scientifica allo sviluppo di strategie terapeutiche innovative. Il primo importante successo nella cura dell'infezione da HIV-1 è stato ottenuto in seguito al trapianto allogenico, in un paziente leucemico HIV positivo, di cellule staminali ematopoietiche (HSCs) naturalmente resistenti all'infezione a causa di una mutazione a livello del corecettore virale CCR5. Tuttavia, il potenziale rischio di rigetto e la difficoltà di reperire donatori compatibili, non consentono l'adozione diffusa di questo approccio. Il risultato ottenuto supporta, invece, l'idea che la modificazione genetica delle HSC, che rappresentano i precursori di tutte le cellule coinvolte nella patogenesi dell'infezione da parte di HIV-1, possa generare un sistema immunitario permanentemente resistente al virus. Negli ultimi anni sono stati sviluppati diversi approcci di terapia genica finalizzati all'espressione di geni anti-HIV-1 nelle HSC. Tra questi, gli approcci basati sulla tecnica dell'RNA interference (RNAi) rappresentano un potente strumento in grado di inibire la replicazione virale.

Partendo da questi presupposti, lo scopo del presente studio consiste nello sviluppo di vettori lentivirali esprimenti small interfering RNA (siRNA) in grado di inibire la replicazione di HIV-1 e di testarne l'efficacia in cellule primarie umane fisiologicamente rilevanti per l'infezione virale, tra cui macrofagi e linfociti T CD4+. L'obiettivo ultimo del più ampio progetto di ricerca, in cui si inserisce questo lavoro, è l'impiego dei vettori antivirali più efficaci per la manipolazione genetica di HSC derivanti da pazienti HIV positivi affetti da linfoma. Questi pazienti rappresentano, infatti, un'opportunità unica per valutare una terapia anti-HIV-1 basata sull'impiego di HSC ingegnerizzate in un contesto clinico eticamente accettabile, poichè sono spesso sottoposti a trapianto di HSC.

A causa dell'elevato tasso di mutazione associato alla replicazione di HIV-1, sono necessarie strategie terapeutiche combinatorie al fine di ridurre il rischio di insorgenza di resistenze. Pertanto, sono state selezionate le seguenti molecole in grado di inibire diverse fasi del ciclo biologico virale: due short hairpin RNA, codificanti un singolo siRNA diretto contro il trascritto del gene cellulare CCR5 (shCCR5) o del gene virale *vif* (shvif) e una long hairpin RNA, codificante due siRNA diretti contro il trascritto comune del primo esone dei geni virali *tat* e *rev* (lhtat/rev).

Nella prima parte del lavoro, sono stati ottenuti vettori codificanti le singole unità trascrizionali esprimenti shCCR5, shvif e lhtat/rev sotto il controllo di diversi promotori umani della polimerasi III, tra i quali U6, 7SK e H1. È stata, quindi, valutata l'attività di silenziamento genico dei singoli siRNA, allo scopo di identificare la migliore combinazione di promotore-siRNA per il successivo sviluppo di approcci antivirali combinatori. In seguito, gli siRNA sopradescritti sono stati clonati tutti all'interno di uno stesso vettore sottoforma di unità trascrizionali indipendenti. In particolare, al fine di ottimizzare il *design* dei vettori, sono state ottenute multiple piattaforme combinatorie, che differiscono l'una dall'altra per i promotori che guidano l'espressione degli siRNA e per la posizione delle unità trascrizionali. Poiché la presenza di promotori multipli potrebbe causare la saturazione del *pathway* cellulare di biogenesi dei microRNA, è stata sviluppata una strategia combinatoria alternativa, basata sull'impiego di extended shRNA (e-shRNA). Questa molecola è in grado di esprimere sotto il controllo di un singolo promotore i tre siRNA contro i trascritti dei geni CCR5, *vif* e *tat*/*rev*. Lo studio dell'attività antivirale delle diverse piattaforme combinatorie ha portato all'identificazione di due vettori (U6shCCR5-7SKshvif-H1lhtat/rev e H1e-shRNA) in grado di inibire efficientemente la replicazione di HIV-1 sia in linee cellulari, che in cellule primarie umane, in assenza di citotossicità.

Nel complesso, i risultati ottenuti evidenziano aspetti importanti che devono essere presi in considerazione per lo sviluppo di approcci combinatori basati su RNAi finalizzati all'inibizione della replicazione di HIV-1. Il presente studio ha portato, inoltre, all'identificazione di nuove piattaforme combinatorie anti-HIV-1 che potrebbero essere testate in futuri studi clinici, una volta accertata la loro efficacia e sicurezza *in vivo* nel modello animale.

3. ABSTRACT

Acquired immunodeficiency syndrome (AIDS) remains one of the most important global public health concern. Although antiretroviral therapy has improved survival in HIV-1-infected patients, drugs cannot eradicate the virus and are associated with toxicity and resistance. Gene therapy holds considerable promise as an alternative or complementary strategy for the treatment of HIV-1 infection and, in this context, RNA interference (RNAi)-based strategies represent one of the most powerful approaches. Stable expression of short hairpin RNAs (shRNAs) targeting viral genes or cellular co-factors in virus target cells can render them resistant to infection. In this study, lentiviral vectors expressing shRNAs targeting the viral genes *vif*, *tat*/*rev* and the cellular gene CCR5 were developed. To account for HIV-1 variability, we adopted combinatorial RNAi approaches based on the simultaneous expression of the different shRNAs either from distinct promoters or as an extended shRNA. The biological activity and safety of the developed vectors were investigated both in cell lines and in human primary cells. Our results showed that vector efficacy is influenced by several constraints, depending on the adopted combinatorial platform. Among these, promoter selection, relative position of the shRNA cassette within the vector framework and presence of repeated sequences turned out to be key factors to be considered. Importantly, we identified new effective anti-HIV-1 RNAi-based platforms that provided robust protection against viral infection. Overall, these data confirmed that combinatorial RNAi is a feasible approach to counteract HIV-1 replication, highlighting some important strengths and pitfalls of different strategies used for the delivery of multiple RNAi effectors. These findings might contribute to the development of a reliable HIV gene therapy approach for future clinical applications.

4. INTRODUCTION

4.1 The human immunodeficiency virus type I

Acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus type1 (HIV-1) infection remains one of the most important global public health threats (Ringpis *et al.*, 2012). Worldwide, more than 35 million individuals are infected with HIV-1 and each year approximately 2 million people are newly infected with no effective vaccine available in both developed and underdeveloped countries (Global Report UNAIDS, 2013).

HIV-1 is a lentivirus, member of the viral family *Retroviridae*, that was first discovered in 1983 (Barré-Sinoussi *et al.*, 1983). Retroviruses are characterized by their use of viral reverse transcriptase and integrase enzymes for stable insertion of viral genomic information into the host genome (Terwilliger *et al.*, 1990). The *lentivirus* appellative refers to the long period of time elapsing between the initial infection and the onset of the disease, that can protract over a period of months or even years.

The HIV-1 genome consists of two linear positive-sense single-stranded RNA molecules of approximately 9 kb in length that encode nine proteins (Figure 4.1A). The three largest open reading frames encode the major structural and enzymatic proteins: Gag, Pol and Env. The *gag* gene encodes viral core proteins, the *pol* gene encodes a set of enzymes required for viral replication and the *env* gene encodes the viral surface glycoproteins. In addition to these major proteins, the HIV-1 genome encodes the regulatory proteins Tat and Rev, which activate viral transcription and control the nuclear export of viral transcripts, respectively, and the accessory proteins Vif, Vpr, Vpu and Nef, that are essential for viral infectivity and spreading during *in vivo* infection (Feng and Holland, 1988; Malim *et al.*, 1989). Besides the protein-encoding regions, *cis*-acting elements are interspersed throughout the HIV-1 genome. Genes are flanked by regulatory sequences, consisting of repeat elements (R) followed by 5' unique elements (U5) at the 5' terminus of the RNA genome, and of 3' unique elements (U3) followed by repeat elements (R) at the 3' terminus of the RNA genome. During the reverse transcription process, these sequences undergo duplication giving rise to identical

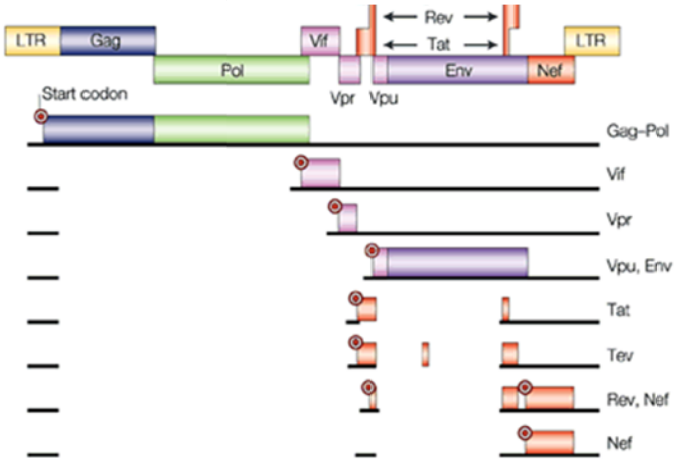
5’ and 3’ long terminal repeats (LTR) consisting of U3-R-U5 sequences at the DNA genome termini. The two LTRs contain signals important for provirus integration into the host genome (*att* repeats), enhancer/promoter sequences, the Tat protein binding site (transactivation response element, TAR) and the polyadenylation signal (polyA). Other *cis*-acting sequences within the HIV-1 genome include the primer binding site (PBS), to which tRNALys binds to initiate reverse transcription, the viral RNA packaging/dimerization signals (Ψ and DIS), the central polypurine tract (cPPT) and the central termination sequence (CTS), that lead to the formation of a three-stranded DNA structure called the central DNA Flap during reverse transcription. In addition, there are the Rev responsive element (RRE), which represents the binding site for the Rev protein, and the purine-rich region (polypurine tract, PPT), that provides a second primer for the initiation of plus strand DNA synthesis by reverse transcriptase (reviewed by Pluta and Kacprzak, 2009).

The 5’ LTR drives transcription of an initial genome-length RNA that also acts as an mRNA for translation of the viral Gag and Pol proteins. The Gag precursor protein (Pr55) is proteolytically cleaved by viral protease to yield matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7), p6 and two spacer peptides (p1 and p2). The Pol protein is produced as a Gag-Pol precursor (Pr160) following a -1 frameshift event, required as the sequences coding for the Gag and Pol proteins are in different reading frames. The frameshifting occurs at an approximate rate of one Gag-Pol for every twenty Gag molecules synthesized. The Gag-Pol polyprotein is cleaved by the same protease resulting in the p6* transframe (TF) polypeptide and in three viral enzymes: protease (PR, p11), reverse transcriptase with ribonuclease H (RNase H) activity (RT, heterodimer p66/p51 and RNase H, p15) and integrase (IN, p31). The initial transcript is also processed into fully spliced transcripts encoding the Tat and Rev regulatory proteins, as well as the Nef accessory protein. Alternatively, this transcript can be processed into partially spliced mRNAs coding for the three other accessory proteins: Vif, Vpu and Vpr. These partially processed transcripts also contain the *env* ORF for envelope glycoprotein (Env, gp160) translated thanks to the leaky scanning through *vpu* AUG. Proteolysis of the precursor envelope glycoprotein gp160 by furin cellular protease results in the formation of the surface subunit (SU, gp120) and the

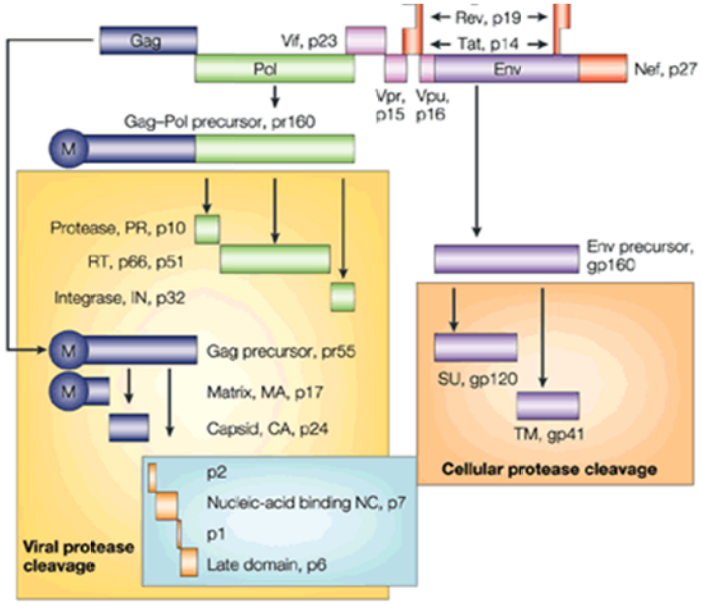
transmembrane subunit (TM, gp41) (reviewed by Pluta and Kacprzak, 2009) (Figure 4.1B).

The lipid-enveloped HIV virion measures approximately 80-120 nm in diameter. The envelope glycoproteins form trimers spiking through the surface of the viral membrane. Just below the viral envelope there is the matrix layer. The viral core, or capsid, is usually bullet-shaped and is made up of the p24 protein. The genetic material is located within the core together with the PR, the RT and the IN enzymes. The core also includes the NC protein, that acts as a nucleic acid chaperone. HIV particles contain hundreds of host cellular proteins important for virus replication and pathogenesis (Chertova *et al.*, 2006) (Figure 4.1C).

A



B



C

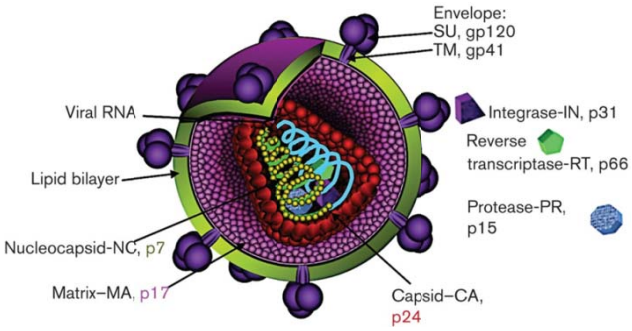


Figure 4.1. Schematic representation of the HIV-1 genome, transcripts and proteins and structure of the virion. (A) Integrated into the host chromosome, the 9-kb viral genome encodes structural (i.e. *gag*, *pol* and *env*), regulatory (i.e. *rev* and *tat*) and accessory (i.e. *vif*, *vpr*, *vpu* and *nef*) genes flanked by LTRs. Viral proteins are synthesized from at least ten transcripts Black lines denote unspliced and spliced transcripts, above which coding sequences are given, with the start codons indicated. (B) HIV proteins. Group-specific antigen (Gag) and Gag–Pol (polymerase) polyprotein precursors are processed by the viral protease into nine subunits: protease (PR), reverse transcriptase (RT), which contains RNAse H, integrase (IN), matrix (MA), capsid (CA), p2, nucleocapsid (NC), p1 and p6 (shown in the yellow box). Env is cleaved by cellular proteases, such as furin, into surface (SU) gp120 and transmembrane (TM) gp41 moieties (shown in the orange box). Tat is the main transcriptional regulator of the long terminal repeat (LTR). Rev is the main nuclear-export protein and it regulates the shift between early and late viral gene expression. The viral-infectivity factor (Vif), viral protein r (Vpr), viral protein u (Vpu) and negative effector (Nef) proteins are known as accessory proteins because they are dispensable for viral growth in some cell-culture systems. Nevertheless, they have essential roles in viral replication and progression to AIDS *in vivo*. (C) Schematic representation of a mature HIV virion detailing the localization of viral proteins and the approximate virion structure (Adapted from Peterlin and Trono, 2003; Steckbeck *et al.*, 2013).

4.2 The HIV-1 life cycle

The natural HIV-1 infection cycle is initiated by attachment of the SU envelope subunit to its CD4 primary receptor exposed on the surface of the host cells (Figure 4.2). After binding to CD4, activated gp120 undergoes a conformational change which produces a binding site for a secondary host cell receptor. The most common strains of HIV-1 utilize a β-chemokine receptor, CCR5, or an α-chemokine receptor, CXCR4, as co-receptors. CCR5 is expressed on monocytes/macrophages, dendritic cells and activated T-lymphocytes, while CXCR4 is mainly expressed on T-lymphocytes (Deng *et al.*, 1996; Choe *et al.*, 1996; Alkhatib *et al.*, 1996; Feng *et al.*, 1996). Viral populations in an infected patient can be categorized as R5-tropic, X4-tropic or dual/mixed [D/M, use of both co-receptors by one virus (dual) and/or a mixture of CCR5-using (R5) and

CXCR4-using (X4) viruses (mixed)] on the basis of the co-receptor usage (Whitcomb *et al.*, 2007). Formation of the gp120/co-receptor/CD4 complexes triggers refolding of the non-covalently associated transmembrane gp41 protein, that inserts an N-terminal hydrophobic fusion peptide into the cellular plasma membrane. The gp41 trimer subsequently folds in, to form a six-helix bundle which couples the viral and cellular membranes promoting their fusion. Once the fusion is initiated, the viral core contents are released into the cytoplasm. The positive sense RNA strand is, then, converted into double-stranded DNA by viral RT within a large ribonucleoprotein structure, called the reverse transcription complex. Reverse transcription requires a specific cellular tRNA annealed to the PBS for the initiation of cDNA synthesis. In the case of HIV-1, tRNALys3 is preferentially selected during virus assembly. The nascent viral DNA binds several viral and cellular proteins to produce the so-called preintegration complex (PIC) that is actively imported into the nucleus through nuclear pores. In the nucleus, the linear provirus cDNA integrates into the DNA of the host cell. Viral IN circularizes the provirus at the *att* repeats and plays a crucial role in its integration into host cell DNA via recombination between the *att* repeats and the integration site. It was shown that integrase is the principal viral determinant of integration specificity, by virtue of its binding with the LEDGF/p75 cellular protein, that mediates the preferential integration target site selection within active transcriptional units (Maertens *et al.*, 2003; Engelman *et al.*, 2008). Once the proviral DNA is integrated, the late phases of the lentiviral life cycle take place. The LTRs, capping the ends of the viral genome, regulate transcription and polyadenylation of viral mRNAs. The LTR at the 5' end of the genome acts as a combined enhancer and promoter for transcription by host cell RNA polymerase II. The LTR at the 3' end of the genome stabilizes these transcripts by mediating their polyadenylation. Basal promoter activity by the 5' LTR is minimal in the absence of the Tat transactivator. Initial transcription in the absence of Tat produces viral mRNAs that are multiply spliced into short transcripts. These short transcripts encode the Tat, Rev and Nef proteins, that facilitate subsequent events in the viral life cycle. Newly synthesized Tat binds to the stem-loop-shaped TAR element on the 5' end of HIV-1 mRNAs, leading to the phosphorylation of the carboxy-terminal domain of RNA polymerase II, that results in dramatic

stimulation of transcriptional processivity. Transcription of viral mRNAs begins at the first nucleotide of the R region in the 5' LTR and polyadenylation occurs at the last nucleotide of R in the 3' LTR. Meanwhile, Rev binds to the RRE element on the viral transcripts to facilitate nuclear export of singly spliced or non-spliced viral transcripts and genome. Singly spliced transcripts encode Env, Vif, Vpr and Vpu, whereas non-spliced viral RNAs are used for translation of Gag and Pol and as the genomic RNAs for progeny viruses. HIV-1 uses the cellular splicing machinery to express its genes. Cellular factors, in concert with both positive and negative *cis* elements within the viral genome, act to promote or repress splicing. These *cis* elements include several splice donor (SD) and acceptor (SA) sequences. Exported viral genomes and proteins are assembled at the plasma membrane in a series of coordinated events, encompassing Gag dimerization and multimerization (thanks to contacts made by the CA, SP1 and NC regions), binding of Gag complexes to genomic viral RNA (through the interaction between the NC domain and the Ψ signal) and migration of the Gag-RNA complexes, Gag, Gag-Pol and Env to the site of assembly. The Gag precursor protein Pr55 plays a central role in this process. Indeed, the MA domain of Gag is responsible for targeting and association with plasma membrane and it also facilitates envelope binding to the site of budding. In addition, Gag-Pol precursor is directed into the assembling particle through its interaction with Gag. HIV-1 completes its replication cycle by budding through the cellular plasma membrane. In T cells, virions are believed to assemble and bud from the so-called lipid rafts, which are plasma membrane microdomains enriched in cholesterol and sphingolipids. Virus egress takes advantage of the interactions between viral late domains in the p6 peptide of Gag and the cellular components of the endosomal sorting complex required for transport (ESCRT), that allow viral and cellular membrane separation (Göttlinger *et al.*, 1991; Strack *et al.*, 2002, 2003). Initially, virus particles are released from the infected cell in an immature form. During or shortly after virus budding, the protease, activated by Gag-Pol multimerization, cleaves the Gag and Gag-Pol polyproteins. This process takes place as an ordered cascade of cleavage reactions, leading to the formation of the inner core of the virions, the development of the conical-shaped core shell and the conversion of the immature virus particle into an infectious virus (reviewed by Ganser-Pornillos *et al.*, 2008).

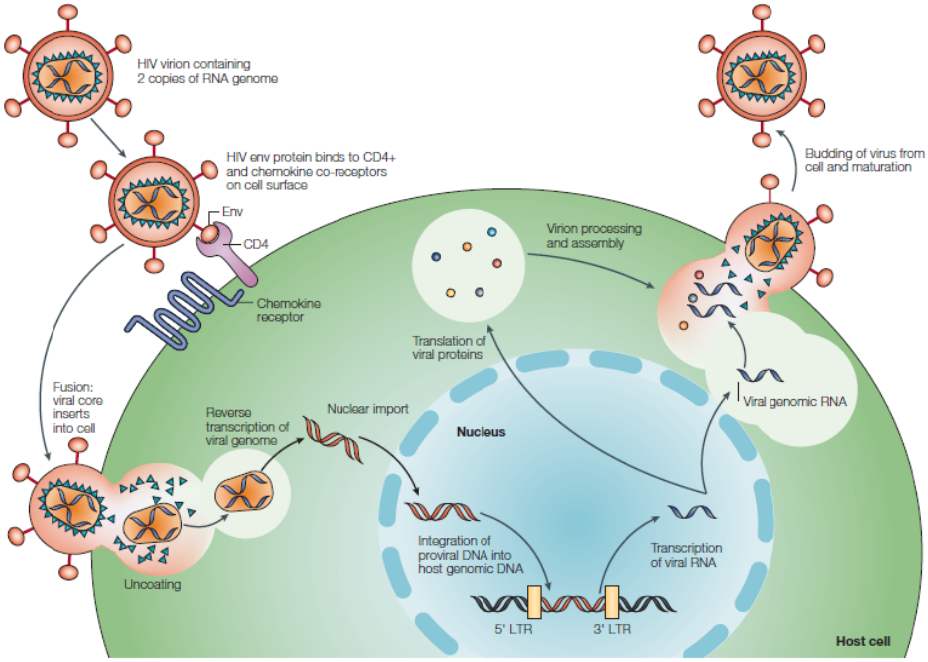


Figure 4.2. Schematic diagram of the HIV-1 life cycle. Upon cell entry, capsid proteins are uncoated, resulting in the release of RNA genome and viral enzymes (PR, RT and IN). The positive sense RNA is converted by RT into double-stranded DNA in the cytoplasm, imported into the nucleus and integrated into the host genome. After transcription, viral mRNAs are processed by cellular machinery. During the early viral life cycle, only fully spliced viral mRNAs (i.e. tat, nef and rev) can be exported from the nucleus to the cytoplasm. After Rev is synthesized, the Rev protein is imported into the nucleus and singly spliced (i.e. vif, vpr, vpu and env) and unspliced (i.e. gag and gag-pol) mRNAs, which contain RRE as a *cis*-element, are exported from the nucleus through interaction with the Rev protein. Once the viral mRNAs are synthesized, viral genome and proteins are assembled at the plasma membrane. New HIV particles are then released from the host cell. Immediately after virus budding, the multimerization of Gag and Gag-Pol activate the viral PR, which leads to the structural rearrangements and gives rise to the mature infectious virions (Adapted from Rambaut *et al.*, 2004).

4.3 Natural history of HIV-1 infection and antiretroviral therapy

HIV-1 is transmitted by contact with infected biological fluids (blood, sperm, vaginal secretions) from infected individuals. The canonical transmission routes are sexual (homo- and hetero-) intercourse; use of infected syringes, typically in drug abusers; vertical transmission from mother to child, with infection occurring during delivery; infected blood or hemoderivatives and occasional wounding with infected material, typically in health workers. In the current pandemics in Africa and Asia, transmission is mainly heterosexual, or vertical in children.

After initial contact, the virus is transported into the secondary lymphoid organs, where infection of CD4+ cells (T lymphocytes and monocytes/macrophages) occurs. A phase of rapid viral replication follows, during which viral load (viremia and the number of infected cells) increases exponentially in blood and in lymphoid organs. This phase of primary infection can be asymptomatic; however, 30-70% of infected individuals experience an acute syndrome, characterized by fever, fatigue, lymphoadenomegaly, maculo-papular cutaneous eruption, and, in a few cases, neurological involvement. The rapid viral replication stimulates a robust immune response, with the generation of neutralizing antibodies and cytotoxic T lymphocytes (CTLs), recognizing and destroying the infected cells. This response curtails viral infection and markedly decreases viral load in patients. However, in contrast to other acute viral diseases, the virus is not eradicated from the organism, since it persists in its integrated, proviral DNA form in a reservoir of latently infected cells. These are mainly CD4+ T memory lymphocytes (CD45+ R0+), which do not proliferate and are metabolically inactive. Since these cells do not transcribe the viral genome, no viral protein is expressed and the cells remained unrecognized by the CTLs. A long period thus begins in which the virus replicates in metabolically active cells (activated T lymphocytes and macrophages), but remains latent in inactive cells. The immune system is unable to eradicate the infection, partly because of this latency phenomenon and partly because the replicating fraction of the virus continuously mutates its sequence and, thus, generates mutants escaping neutralizing antibodies and CTLs. These mutant variants are continuously selected *in vivo* upon pressure of the immune response. This condition can last several years, in which the patient is asymptomatic or paucisymptomatic, but shows relevant levels of plasma viremia and is, thus, infective. This asymptomatic phase of the disease eventually exhausts the immune function, mainly because of the progressive decline in the CD4+ T cells, which are the main targets for infection. Since these cells provide an essential helper function to both antibody production and CTL function, the patients progressively become immunodeficient. This acquired immunodeficiency syndrome (AIDS) phase is characterized by recurrent infections, due to normally non-pathogenic microorganisms, and by the development of malignant tumors. The infectious diseases of AIDS patients include recurrent pulmonitis, cerebral

toxoplasmosis, cryptosporidiosis, cutaneous infections and meningitis. The malignant tumors of these patients include Kaposi's sarcoma and non-Hodgkin's lymphomas. In the absence of therapy, the median life expectancy after HIV-1 infection is about 9.5 years (Vergis *et al.*, 2000; Ho and Bieniasz, 2008, Cohen, 2011).

The treatment of HIV-1 infection was revolutionized in the mid-1990s by the development of inhibitors of the viral reverse transcriptase and protease enzymes, and by the introduction of drug regimens that combined these agents to enhance the overall efficacy and durability of therapy. The advent of combination therapy, also known as highly active antiretroviral therapy (HAART), was seminal in reducing the morbidity and the mortality associated with HIV-1 infection and AIDS (Arts and Hazuda, 2012). The U.S. Food and Drug Administration approved 37 antiretroviral drugs for the treatment of HIV infection up to October 2013. These include nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, entry inhibitors (CCR5 co-receptor antagonist) and integrase strand transfer inhibitors (www.fda.gov). Typical HAART regimens include a backbone of two NRTIs and a base of either a PI or NNRTI. New classes of drugs like entry inhibitors and integrase inhibitors have also emerged in clinical practice (Margolis *at al.*, 2013). Combination antiretroviral therapy dramatically suppresses viral replication and reduces the plasma HIV-1 viral load, resulting in a significant reconstitution of the immune system. With proper adherence, HAART can suppress viral replication for decades, increasing the life expectancy of the HIV-infected individual (Arts and Hazuda, 2012).

However, HAART cannot eliminate HIV-1 infection. Cessation of even prolonged HAART regimens results in viral load rebound to pre-therapy levels, indicating the inability of chemotherapy to eradicate HIV-1 infection. This failure has been attributed to the presence of a long-lived, stable population of latently infected cells that are not eliminated by the antiviral treatment, since this treatment only targets the replicating fraction of the virus. Several of these cells are long-lived memory T cells that have an integrated proviral DNA, that is kept in a transcriptionally silent state. The persistence of latent HIV-1 reservoirs is at present the principal barrier to the complete eradication of HIV-1 in patients

treated by HAART, making HIV-1 infection a chronic disease for which there is currently no functional or sterilizing cure (Stevenson, 2013). The prospect of maintaining therapy for the lifetime of a patient represents a major hurdle. In addition, HAART is still fraught with important long-term toxicity, including an increased cardiovascular risk. This is mainly due to the use of HIV protease inhibitors, which determine hyperlipidemia and, thus, increased coronary risk. Furthermore, the effect of therapy can be impaired by nonadherence, poor drug tolerability and interactions among antiretroviral agents and other medications, that decrease optimal drug levels. Each of these can lead to virologic failure and the evolution of drug resistance. For all antiretroviral drug classes, drug resistance has been documented in patients failing therapy, as well as in therapy-naive patients infected with transmitted, drug-resistant viruses. Considering that the virus continues to evolve and escape, with even the most effective therapies, new HIV-1treatments will always be needed (Arts and Hazuda, 2012).

4.4 Gene therapy of HIV-1 infection

Gene therapy has potential as an alternative or complementary treatment strategy to HAART for HIV-infected individuals. Gene therapy can contribute to the treatment of HIV-1 infection by at least three different modalities: by rendering the target cells resistant to infection or viral replication (intracellular immunization), targeting either the viral proteins or the viral RNAs; by inducing the selective activation of suicide or antiviral genes upon HIV-1 infection or by activating the immune system to recognize and destroy the infected cells.

The intracellular immunization approach intends to make HIV-1 target cells resistant to viral infection by introducing anti-HIV-1 genes (Baltimore, 1988). Over the past 20 years, researchers have developed numerous gene-based reagents capable of inhibiting HIV-1 infection by intracellular immunization (Figure 4.3). These have included the expression of intracellular antibodies to viral proteins; antisense RNAs, that inhibit reverse transcription, viral gene expression, processing or translation of HIV-1 RNAs; mutant HIV-1 structural or regulatory genes, with dominant repressor activity (including Rev, Gag and Tat); RNA decoys, that inhibit HIV-1 transcription (multimeric TAR) and processing

(multimeric RRE); ribozymes, to catalytically cleave and inactivate the various HIV-1 RNA species, and small interfering RNAs (siRNAs), that downregulate HIV-1 RNA sequences (reviewed by Hoxie and June, 2012). A different strategy targeting HIV-1 entry was explored by Egelhofer and colleagues (Egelhofer *et al.*, 2004), that developed an HIV-1 fusion inhibitor, termed C46, derived from the C-terminal domain of HIV gp41. C46 can be stably expressed into virus target cells, where it blocks HIV fusion by binding to gp41. In addition, a more recent novel approach to disrupt the CCR5 co-receptor gene was developed by using engineered zinc finger nuclease proteins (ZFNs) (Perez *et al.*, 2008; Holt *et al.*, 2010; Wilen *et al.*, 2011; Li *et al.*, 2013). ZFNs are comprised of custom-made zinc finger DNA binding domains fused to an endonuclease domain that generates a double-strand break at a specific DNA target site. When these double-strand breaks are repaired, deletions and insertions can be introduced at the site of cleavage through a non-homologous end joining (NHEJ) cellular DNA repair mechanism.

Furthermore, studies of the underlying mechanisms of host restriction factors are expanding the repertoire of possible molecular gene therapy approaches against HIV-1. In this context, attention has been devoted to the TRIM5α protein. TRIM5α is a cellular protein that plays an important role in the restricted host range of HIV-1 (Stremlau *et al.*, 2004). Indeed, TRIM5α of old world monkeys, such as rhesus macaques, inhibits HIV-1 infection by disrupting the uncoating of the viral capsid in the cytoplasm, while the human orthologue is permissive. Only one or two aminoacid differences between the human and rhesus TRIM5α are responsible for the opposite behavior of the two proteins. These evidences lent support for the use of chimeric human-rhesus TRIM5α variants (Anderson *et al.*, 2009) or engineered human variants to block HIV infection (Sayah *et al.*, 2004; Pham *et al.*, 2010; Neagu *et al.*, 2009, Chan *et al.*, 2012). All of these anti-HIV genes mediated efficient HIV-1 inhibition in various experimental settings and some of these were also tested in clinical trials. Other host HIV-1 restriction factors that could be potentially utilized as gene therapy reagents are APOBEC3G, APOBEC3F and Tetherin (reviewed by Kitchen *et al.*, 2011). The APOBEC3 proteins are packaged in the virion and block reverse transcription by deaminating nascent viral cDNA, while Tetherin prevents viral budding from the

plasma membrane. HIV-1 is naturally equipped with accessory proteins to counteract these host restriction factors, but several researches developed APOBEC3G and Tetherin variants which are resistant to viral countermeasures.

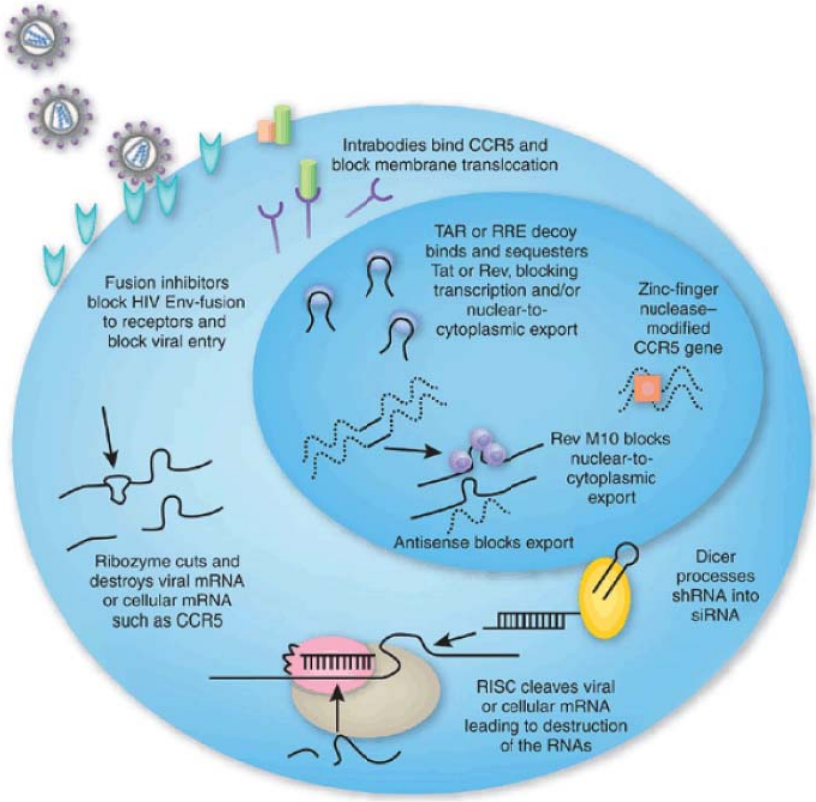


Figure 4.3. Inhibitory agents used in HIV gene therapy trials. Protein-based inhibitors can be directed toward cellular or viral targets and have included fusion inhibitors, intrabodies, zinc finger nucleases and dominant negative inhibitors (Rev M10). Alternatively, a number of RNA-based approaches, including decoys, antisense RNAs, ribozymes and si/shRNAs have been described. An advantage of these latter inhibitors is that, in contrast to protein-based inhibitors, they do not elicit adaptive T-cell responses and are unlikely to be immunogenic. However, RNA approaches can potentially have off-target toxicity because of activation of innate immune responses and competition with endogenous RNA functions (Adapted from Jacobson, 2013).

4.5 Potential cellular targets for anti-HIV-1 gene therapy

Anti-HIV-1 gene(s) can be introduced into hematopoietic stem cells (HSCs) and/or T lymphocytes to provide a population of cells that is protected from viral infection. Both HSC- and T-cell-directed approaches have shown promise (Hoxie and June, 2012).

The most attractive target cell for anti-HIV gene therapy is represented by HSCs, as they are capable of self-renewal and differentiation into all hematopoietic lineages. Gene therapy approaches that introduce protective genes against HIV-1 via HSCs can continuously produce their antiviral genes in all differentiated cells, including HIV-1 target cells, such as CD4+ T lymphocytes, macrophages and dendritic cells. Successful replacement of a patient's immune system by gene modified HIV-1 protected cells may have the potential to minimize viral load, as well as to reduce reservoirs of infected and latently infected cells. Newly differentiated protected cells may prevent viral production and spread from persistently infected cells, and may allow the functional restoration of the damaged immune system. If successful, gene therapy through stem cells could free patients from lifelong daily medications (Kitchen *et al.*, 2011).

A typical protocol for *ex vivo* HSCs gene therapy requires cells to be isolated from the bone marrow or mobilized by several rounds of G-CSF (granulocyte colony stimulating factor) injection. The stem cells are collected by apheresis and the CD34+ cells enriched. Following culture *ex vivo* in conditions that stimulate cell proliferation, the cells are exposed to a retroviral vector expressing the therapeutic gene(s) and, then, infused back into the patient after a few days. Infusion usually takes place following administration of a pharmacological conditioning regimen, that eliminates the endogenous bone marrow progenitors and favors engraftment of the transplanted cells. If the gene-corrected cells have a selective growth advantage compared to the unmodified cells, full reconstitution of the immune cell compartments is obtained even from a few engrafted transduced progenitors cells, and this may occur without conditioning (Naldini, 2011). A potential drawback of this procedure is that manipulation of HSCs is technically challenging, as these cells are difficult to be maintained in culture without losing viability or undergoing differentiation (Hoxie and June, 2012) (Figure 4.4).

Considering that HIV infection causes a severe depletion of CD4+ T lymphocytes, genetic modification of T cells to inhibit viral replication cycle offers an alternative to HSC manipulation. However, the issue of whether the addition of engineered CD4+ T-cell may preserve their immune function allowing long-term persistence is challenging. On the other hand, mature T cells appear to be less

susceptible than HSCs to genotoxicity from integrating viral vectors (Hoxie and June, 2012).

For CD4+ T cell gene therapy, patients are apheresed and the T cell population is enriched. The CD8+ cells are depleted by antibody treatment and the CD4+ cells are stimulated to divide. These cells are transduced with the viral vector harboring the anti-HIV genes and the transduced cells are expanded *ex vivo* prior to infusion back into the patient (Scherer and Rossi, 2011) (Figure 4.4).

Given the chronic but typically manageable nature of HIV infection with HAART, the clinical and ethical criteria for patient selection in gene therapy trials will need to be carefully considered. Notably, patients who develop AIDS-related malignancies, particularly lymphomas, a subset of which will require autologous HSC transplants, represent a unique cohort in which gene therapy approaches can be evaluated, because their HSCs are mobilized and harvested before chemotherapy. This provides both an opportunity to engineer HSCs to be resistant to HIV infection and to increase the chances of engraftment of the modified cells following a myeloablative conditioning regimen. Moreover, anti-lymphoma chemotherapy in this setting may have the added benefit of targeting HIV reservoirs (Hoxie and June, 2012).

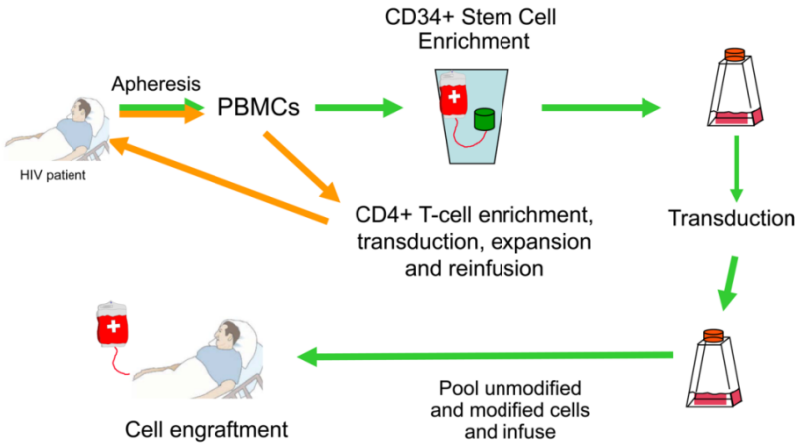


Figure 4.4. Representation of autologous stem and T-cell gene therapy for treatment of HIV-1 infection. CD34+ progenitor cells are mobilized, collected, transduced and infused into patients. This process requires some myeloablation to create space for stem cell engraftment. The T-cell approach requires isolation of PBMCs, removal of CD8+ cells and transduction of CD4+cells followed by *ex vivo* expansion and infusion into the patients (Adapted from Zhou and Rossi, 2011).

4.6 HIV-1 gene therapy trials

Several anti-HIV-1 gene therapy protocols have been tested in clinical trials. Most clinical trials were phase I studies aimed at evaluating the safety and feasibility of anti-HIV-1 gene-transduced autologous CD4+ T lymphocyte and hematopoietic stem/progenitor cell transplantation in patients. In early trials, dominant negative proteins (RevM10), RRE decoy, or anti-HIV-1 ribozyme were introduced into patient's CD34+ cells with Moloney murine leukemia virus (MLV)-based gammaretroviral vectors. All of these phase I clinical studies demonstrated safety and feasibility of the procedures. Gene transfer and stem cell transplantation were well tolerated and no significant adverse events have been observed. In all of these trials there were detectable levels of anti-HIV-1 gene expressing cells in patients; however, the gene marking levels were too low to achieve clear therapeutic benefits (reviewed by Kitchen *et al.*, 2011).

Mitsuyasu and colleagues reported the first phase II clinical trial of an anti-HIV-1 gene therapy approach in 2009 (Mitsuyasu *et al.*, 2009). Here the investigators used the MLV-based OZ1 vector containing a gene encoding a ribozyme targeting the HIV-1 overlapping *vpr* and *tat* reading frames. In both phase I and II clinical trials, autologous CD34+ HSCs were transduced and administered without the subject undergoing myeloablation or any form of bone marrow conditioning. The phase I trial reported no serious adverse events related to the gene transfer process or the gene transfer product, and the transgene was detected in peripheral blood cells and bone marrow cells of some patients up to 3 years after a single infusion of the genetically modified cells. In phase II trial, which enrolled 74 subjects, ribozyme DNA and RNA were detectable in 94% of patients, but gradually declined to 7% of patients 100 weeks following treatment. Although the levels of ribozyme DNA and RNA were low, lower viral loads and higher CD4+ cell counts were observed (Mitsuyasu *et al.*, 2011). Overall, these studies demonstrated a proof of concept that anti-*tat*/*vpr* ribozyme transduced autologous HSCs transplanted in humans is safe and has a capability to produce gene modified cells in a large numbers of human subjects (Kitchen *et al.*, 2011).

The first anti-HIV-1 gene therapy clinical study employing a lentiviral vector expressing a combination of antiviral molecules was described by DiGiusto and

colleagues in 2010 (DiGiusto *et al.*, 2010). Four AIDS lymphoma patients undergoing treatment with transplanted HSCs were also given gene-modified CD34+ hematopoietic progenitor cells expressing three RNA-based anti-HIV moieties (*tat*/*rev* short hairpin RNA, TAR decoy, and CCR5 ribozyme). *In vitro* analysis of these engineered cells showed no differences in their hematopoietic potential compared with untransduced cells. Ethical study design required that patients were transplanted with both gene-modified and unmanipulated hematopoietic progenitor cells, obtained from the patient by apheresis. Transduced cells were successfully engrafted in all four infused patients by day 11, and there were no unexpected infusion-related toxicities. Persistent vector expression in multiple cell lineages was observed at low levels for up to 24 months, as was expression of the introduced siRNA and ribozyme. These results support the development of a combinatorial RNA-based cell therapy platform for HIV-1.

Another approach to HIV-1 therapy based on gene transfer employed VRX496, an HIV-1-based lentiviral vector expressing a 937-base antisense gene complementary to HIV-1 *env*. In this context, transgene transcription is under the control of the native HIV-1 LTR, thus infection with HIV-1 and the resulting Tat expression transactivate VRX496 to up-regulate the antisense RNA expression. Because VRX496 retains HIV-1 *cis*-acting elements required for replication, the vector can potentially be mobilized by HIV-1 infection and spread to new CD4+ T cells. Levine and co-workers reported results after a single infusion of VRX496-containing CD4+ T cells in patients failing HAART (Levine *et al.*, 2006). The infusion was safe and associated with improved CD4+ T cell counts, persistent gene transfer and no evidence of insertional mutagenesis. Given these results, investigators hypothesized that multiple infusions of gene-modified cells in earlier stage patients with well-controlled viremia would improve the persistence of VRX496 and enhance the therapeutic effect. Results of this latter clinical trial reported the safety and tolerability of multiple infusions of vector-modified autologous CD4+ T cells. A significant decrease in viral load set point was observed after discontinued antiretroviral therapy. In addition, it was found that expression of the antisense sequence in gene-modified cells exerted genetic pressure on HIV-1, causing production of replication-impaired virus. The

engraftment half-life of vector-modified CD4+ T cells in the blood was approximately 5 weeks, with stable persistence in some patients for up to 5 years. No evidence of clonal selection of vector-transduced T cells or integration enrichment near oncogenes was detected. This early-phase study pointed out that gene-modified T cells have the potential to decrease the fitness of HIV-1 and conditionally replicative lentiviral vectors have a promising safety profile in T cells (Tebas *et al.*, 2013).

These and other trials have shown the safety of the procedure and of the anti-HIV-1 agents themselves, as well as the feasibility of the approach in which autologous HSCs and/or CD4+ T cells are taken from the subject, genetically manipulated and given back to the subject. Areas for further focus appear to be maximizing the number of the infused gene-tranduced cell; maximizing the engrafment, proliferation and differentiation of this genetically modified cells, possibly incorporating partial myeloablation; increasing the effectiveness of the used anti-HIV-1 gene(s) and automation of the cell processing procedure (Mitsuyasu *et al.*, 2011).

4.7 RNA interference as an anti-HIV-1 therapeutic

Since the first description of RNA interference (RNAi) in 1998 (Fire *et al.*, 1998), it has rapidly become one of the methods of choice for gene function analyses and it is being exploited for therapeutic applications. To date, preclinical studies indicate that RNAi is the most potent RNA-based inhibitory mechanism available for therapeutic application (Hoxie and June, 2012).

HIV-1 was one of the first infectious agents targeted by RNAi as a result of the virus relatively well-understood life cycle and pattern of gene expression. RNAi induction has been shown to be highly effective in inhibiting HIV-1 (Lee *et al.*, 2005; Liu *et al.*, 2007; Sano *et al.*, 2008). RNAi triggers have been used to target virtually all the HIV-encoded RNAs in cell lines, including *tat*, *rev*, *gag*, *pol*, *nef*, *vif*, *env*, *vpr* and the LTRs. Indeed, each step in the replicative cycle of HIV-1 could be considered as target for RNAi-based therapeutic intervention. Other studies have shown a host of other viruses, including the hepatitis B virus, hepatitis C virus, poliovirus and respiratory syncytial virus to be targeted by

RNAi (reviewed by Leonard and Schaffer, 2006). However, it has been shown that knockdown efficacy of an RNAi effector varies according to its sequence and target site on RNA and hence results in limited number of highly potent therapeutic agents (Tyagi *et al.*, 2011).

RNAi is an evolutionary conserved mechanism that triggers sequence-specific inhibition of complementary mRNAs in eukaryotes. In mammals, RNAi is a post-transcriptional gene silencing mechanism that functions to regulate gene expression via small hairpin-like double stranded RNA (dsRNA) molecules called microRNAs (miRNAs) (Figure 4.5). More than 1000 human miRNAs have been identified, which are estimated to regulate the expression of at least 30% of human genes (Liu and Berkhout, 2011). miRNAs are expressed from polymerase II promoters as primary miRNA transcripts (pri-miRNAs), forming distinctive imperfect hairpin structures. Pri-miRNAs are first processed by a Drosha complex cleaving ~22 bp back from the stem-loop junction, to release a 60-80 nucleotide hairpin (pre-miRNA) and, then, exported from the nucleus to the cytoplasm by the nuclear Exportin-5 protein. In the cytoplasm, Dicer next cleaves from the opposite end, removing the loop to release a small RNA duplex of ~21 bp with characteristic 2-nt 3' overhangs (the mature miRNA). The duplex is then loaded into the RNA induced silencing complex (RISC) and unwound into the effector guide strand and the passenger strand. The guide strand is selected according to thermodynamic stability at the ends of the duplex. This strand directs RISC to bind target RNA within the 3' untranslated region, resulting in translational repression, mRNA destabilization or a combination of both. Near-perfect base pairing of the miRNA with the mRNA results in cleavage-mediated inactivation of the target mRNA. The targeted mRNA is translocated to cellular processing (P)-bodies where storage, de-adenylation, de-capping and degradation take place (McIntyre *et al.*, 2011; Liu and Berkhout, 2011). In invertebrates and plants, RNAi provides an innate defence mechanism against invading RNA viruses, as the introduction of dsRNAs into the cells of these organisms leads to the processing of the dsRNA molecules into siRNAs that serve as guides for enzymatic cleavage of complementary RNAs (Yu *et al.*, 2002).

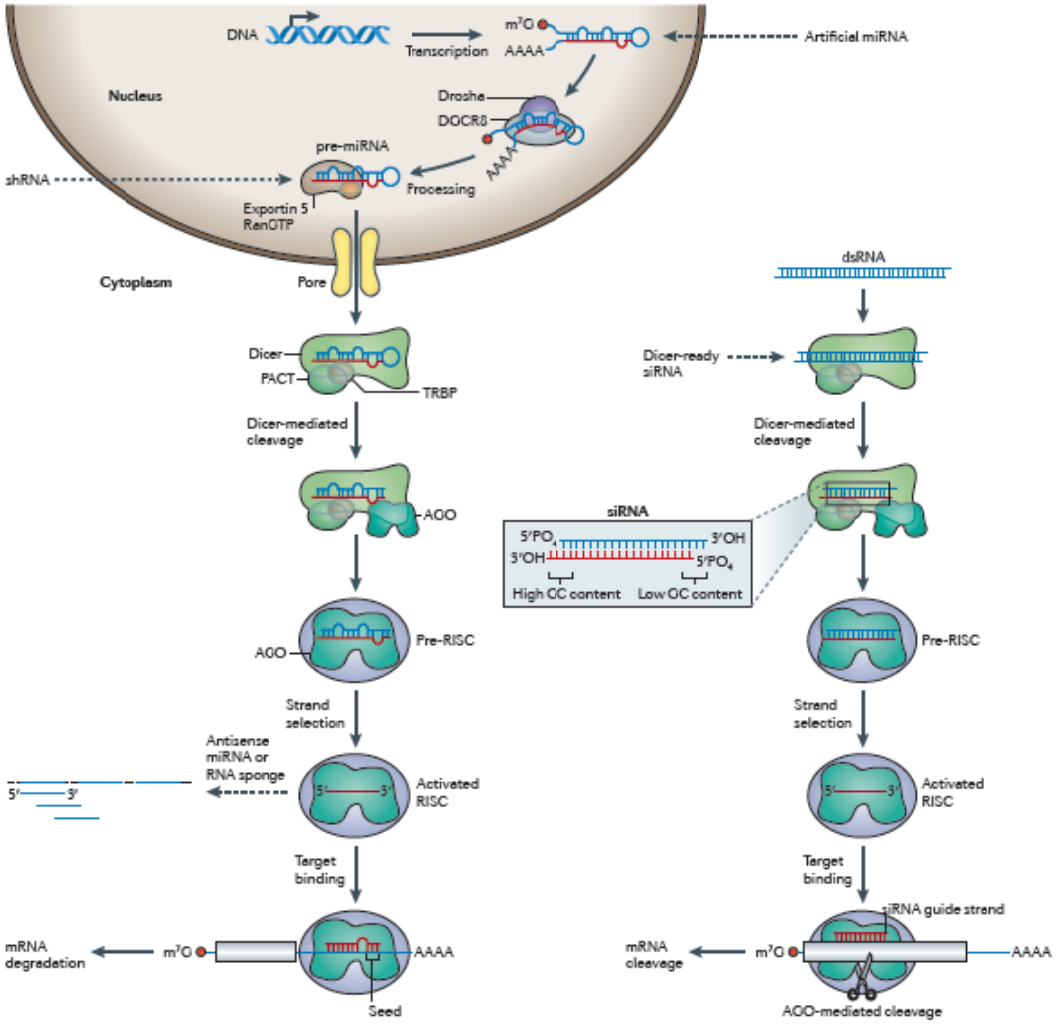


Figure 4.5. The miRNA and siRNA pathways of RNAi in mammals. Primary microRNAs (pri-miRNAs) are transcribed by RNA polymerases and are trimmed by the microprocessor complex (comprising Drosha and microprocessor complex subunit DCGR8) into ~70 nucleotide precursors, called pre-miRNAs (left side of the figure). miRNAs can also be processed from spliced short introns (known as mirtrons). Pre-miRNAs contain a loop and usually have interspersed mismatches along the duplex. Pre-miRNAs associate with exportin 5 and are exported to the cytoplasm, where a complex that contains Dicer, TAR RNA-binding protein (TRBP; also known as TARBP2) and PACT (also known as PRKRA) processes the pre-miRNAs into miRNA–miRNA* duplexes. The duplex associates with an Argonaute (AGO) protein within the precursor RNAi-induced silencing complex (pre-RISC). One strand of the duplex (the passenger strand) is removed. The mature RISC contains the guide strand, which directs the complex to the target mRNA for post-transcriptional gene silencing. The 'seed' region of a miRNA is indicated. Long dsRNAs (right side of the figure) are processed by Dicer, TRBP and PACT into small interfering RNAs (siRNAs). siRNAs are 20–24-mer RNAs and harbour 3′OH and 5′ phosphate (PO_4) groups, with 3′ dinucleotide overhangs. Within the pre-RISC complex, an AGO protein cleaves the passenger siRNA strand. Then, the mature RISC, containing an AGO protein and the guide strand, associates with the target mRNA for cleavage. The thermodynamic stability of the siRNA terminal sequences will direct strand loading (Adapted from Davidson and McCray, 2011).

RNAi can be co-opted by delivering synthetic siRNAs duplexes of 19-21 bp, that, mimicking the mature miRNAs, are loaded directly into RISC, to mediate post-transcriptional silencing of their target (McIntyre *et al.*, 2011). A constant supply of siRNAs is required to combat chronic infections like HIV-1, thus much of the anti-HIV RNAi research is focused on the development of RNAi triggers that can be stably expressed within target cells as gene therapy strategy (Schopman *et al.*, 2010). To this end, a short hairpin RNA (shRNA) consisting of the sense and antisense sequences of an siRNA connected by a loop of unpaired nucleotides can be expressed by viral vectors (Figure 4.6A). Following transcription, shRNAs are exported to the cytoplasm and processed by Dicer and, finally, they engage the RISC complex via the normal miRNA biogenesis pathway. shRNA design often occurs by the addition of a loop to an optimally designed siRNA core. The hairpin loop may be an important determinant of the shRNA activity (Schopman *et al.*, 2010). The most extensively adopted loop sequence is a 9-mer nucleotide sequence (TTCAAGAGA) published by Brummelkamp and co-workers in 2002 (Brummelkamp *et al.*, 2002).

Expression of shRNAs is mostly driven by RNA polymerase III promoters, including the small nuclear RNA U6 promoter, the RNase P RNA H1 promoter and tRNA promoters, because of their natural function in the production of small cellular transcripts (Liu and Berkhout, 2011). These promoters are compact, active in many tissues, strong and usually generate a huge amount of transcription products that are presumably processed directly by Dicer. Another advantage of using polymerase III promoters is that the sequence of the generated siRNA can be controlled because the transcription starts from the +1 position of the promoter transcription unit and termination occurs within a stretch of uracils in the terminator sequence, facilitating the generation of double stranded shRNA with 3' overhangs, that is essential for Dicer processing (Manjunath *et al.*, 2009).

The original shRNA design has been further optimized by embedding the hairpins in a microRNA-like context via inclusion of structural motifs of pri-miRNAs (Figure 4.6B). In this approach, an siRNA is inserted at the location of the mature miRNA in a specific pri-miRNA scaffold. Artificial miRNAs are usually transcribed from an RNA polymerase II promoter, that is the natural promoter of most miRNA genes. The use of RNA polymerase II promoters has some benefits

in that regulatable and tissue-specific variants exist, yet inducible RNA polymerase III systems have also been described (Liu and Berkhout, 2011).

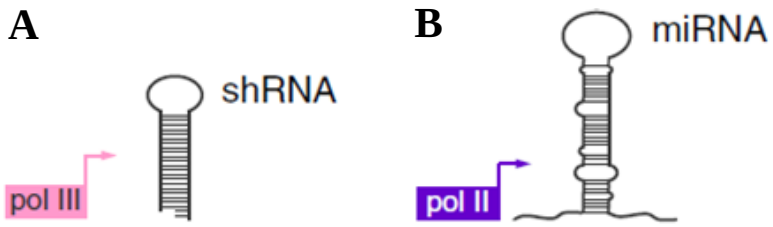


Figure 4.6. Artificial RNAi effectors. (A) Short hairpin RNAs (shRNAs) composed of a perfectly complementary stem with a small hairpin loop are commonly expressed from RNA polymerase III promoters, because they allow precise initiation and termination of transcription. (B) Artificial miRNAs resemble the natural miRNAs with their characteristic features, including loops, internal mismatches, bulges and flanking sequences (Adapted from Liu and Berkhout, 2011).

4.8 Combinatorial RNAi strategies

The use of a single anti-HIV-1 gene may not be sufficient to protect cells long-term from infection, due to the high mutation rate of HIV-1. Indeed, development of viral resistance is a common setback with HIV-1 therapies, because of the generation of viral escape mutants (Zhou and Rossi, 2011). Thus, similar to combination approaches with small-molecule drugs, effective gene therapy applications against HIV-1 disease will require a combination of multiple reagents directed against the virus (Scherer and Rossi, 2011).

A number of different escape routes have been described when inhibiting HIV-1 replication by means of RNAi-based approaches. Firstly, a point mutation in the target sequence can reduce the complementarity with the shRNA inhibitor and, thereby, abolish the RNAi-suppression. Secondly, the complete or part of the target region could be deleted, when non-essential viral genes are targeted. Thirdly, resistance-causing mutations were observed outside the target region. These mutations elicit a structural change in the HIV-1 mRNA, thus making the target sequence inaccessible for the RNAi-machinery (Eekels *et al.*, 2011).

In silico studies analyzing the impact of anti-HIV-1 gene therapy provided evidence that four or more shRNAs with targets within viral genes can effectively suppress the spread of infection, while constraining the development of resistance (McIntyre *et al.*, 2009). There are several different methods for co-expressing

multiple RNAi triggers, including: different expression vectors; multiple shRNA-expression cassettes from a single vector; long hairpin RNAs (lhRNAs); extended-short hairpin RNAs (e-shRNAs) and multiple miRNA-embedded shRNAs (Figure 4.7).

The multiple expression cassette strategy is perhaps the most promising and has been used successfully in transient expression studies with cassette combinations ranging from 2 to 7 (ter Brake *et al.*, 2006; McIntyre *et al.*, 2009; Centlivre *et al.*, 2013). However, different promoters should be used, as the presence of repeated sequences might cause recombination within the vector genome during the transduction process, resulting in deletion of one or more cassettes (ter Brake *et al.*, 2008).

Alternatively, lhRNAs or e-shRNAs expressing multiple effective siRNAs from a single promoter can mediate a durable HIV-1 inhibition (Liu *et al.*, 2007, 2009; Sano *et al.*, 2008; Saayman *et al.*, 2008). lhRNAs produce siRNAs targeting adjacent mRNA sites, but the siRNA units are not well-defined and have not been previously singly tested for knockdown activity (Liu and Berkhout, 2011). By contrast, e-shRNAs are based on careful stacking of two or more pre-validated siRNAs. Several constrains regulate the design of the e-shRNAs. It has been demonstrated that hairpin transcripts have un upper size limit for effective production of multiple, functional siRNAs. In general, the hairpins seem to lose activity when they get larger than 66 bp. This decrease in siRNA production could be due to reduced expression, diminished stability, hampered nuclear export, or poor processing into functional siRNAs (Liu *et al.*, 2009). Thus, the design of e-shRNAs is restricted to the expression of three active siRNAs, because addition of a fourth siRNA resulted in a dramatic decrease in hairpin transcript expression. Other relevant aspects to be considered are the spacing between the stacked siRNAs and their positioning along the hairpin stem. Indeed, on the one hand, the exact cleavage site of Dicer is unknown and pilot research is needed to accurately stack the siRNA units, such that the proper inhibitors are made upon Dicer processing (Liu and Berkhout, 2011). Berkhout and colleagues adopted a 3-mer nucleotide spacer sequence between consecutive siRNAs in their successful e-shRNA design (Liu *et al.*, 2009). On the other hand, it has been shown that the siRNAs are produced in a gradient from the base of the hairpin towards the top,

with the most abundant and active one being at the base. This is probably due to a reduced Dicer processing towards the hairpin loop (Liu and Berkhout, 2011).

An alternative combinatorial RNAi strategy is based on the expression of multiple siRNAs from a miRNA polycistron. By mimicking a miRNA cluster, multiple siRNAs can be expressed from one single RNA polymerase II transcript. This approach closely resembles the natural situation where several miRNAs can be expressed in a coordinated manner from a single transcriptional unit. Despite the many positives results with the microRNA approach, this strategy is not yet broadly employed by researchers because of its complex design. For example, the flanking sequences, the position of the siRNA insert within the miRNA hairpin, the miRNA scaffold and the simultaneous co-expression of another miRNA hairpin have all shown to influence RNAi activity (Liu and Berkhout, 2011).

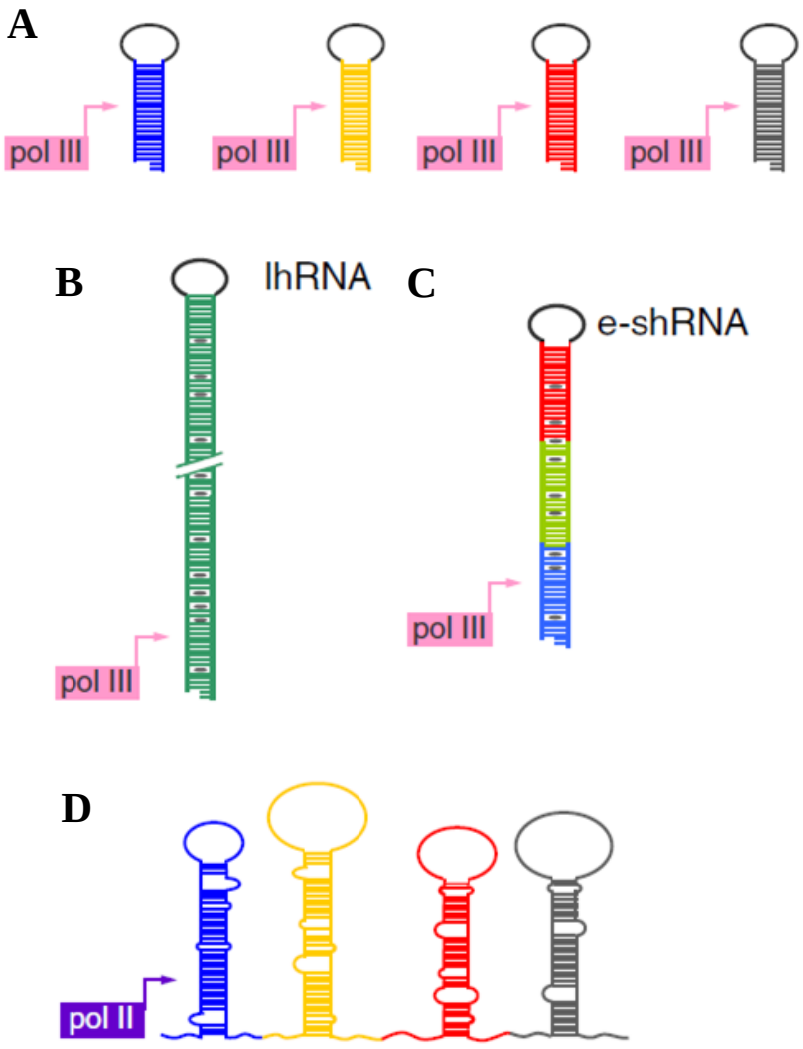


Figure 4.7. RNAi-based combinatorial approaches. (A) Combinatorial RNAi via the expression of multiple shRNAs from independent expression cassettes. (B) Expression of many siRNAs is possible with long hairpin RNAs (lhRNAs). These siRNAs target consecutive mRNA sequences, but none of the siRNA units have been pre-tested for knockdown efficiency. (C) Expression of multiple siRNAs from designed e-shRNAs, that are composed of several active siRNA units. (D) Expression of multiple siRNAs by expression of a miRNA polycistron. By mimicking a miRNA cluster, multiple siRNAs can be expressed from one single RNA polymerase II transcript (Adapted from Liu and Berkhout, 2011).

4.9 Toxicity associated with RNAi triggers expression

The ectopically expressed RNAi effectors can elicit three types of toxicity. Firstly, off-target effects may occur (Snøve and Rossi, 2006). Considering that the majority of off-target effects are caused by small regions of sequence homology between the guide strand of the siRNA and the 3' untranslated region (UTR) of

cellular genes, it is important to scan for such homologies, as well as to ensure that the passenger strand is not loaded to RISC. This latter point can be achieved by designing shRNAs with a thermodynamically less stable 5' end and more stable 3' end, after processing by Drosha and Dicer (Manjunath *et al.*, 2009).

Secondly, ectopic expression of shRNAs can lead to competition for and possibly saturation of endogenous cellular components involved in RNAi. This has been shown for exportin-5, the transport carrier that is needed for nuclear export of endogenous microRNA precursors. In one study, long-term and sustained expression of shRNAs via adeno-associated viral (AAV) vector in the mouse liver resulted in a dose-dependent liver injury and led to mortality at high doses of expression. This morbidity has been attributed to the saturation of exportin-5 by shRNAs, since the liver-derived microRNAs were significantly downregulated in these mice (Grimm *et al.*, 2006). Consistent with this, over expression of exportin-5 could relieve competition with cellular microRNAs (Yi *et al.*, 2005). Another study showed that the polymerase III U6 promoter-processed transcripts accumulate both in the cytosol and nucleus, indicating that both exportin-5 and Dicer might be saturated (Boudreau *et al.*, 2008). Similarly, lentiviral expression of large amounts of shRNAs generated from the U6 promoter resulted in toxicity in primary human T lymphocytes *in vitro*, that could be mitigated by lower level of expression under the control of H1 promoter (An *et al.*, 2006). Therefore, even if potent and sustained shRNA expression is crucial for the successful application of RNAi to therapeutic interventions, caution is necessary, as the amount of expressed shRNAs can be a critical determinant of whether they are toxic.

Thirdly, shRNAs can cause cytotoxicity by triggering type I interferon (IFN) responses, resulting in non-sequence specific degradation of messages (Snøve and Rossi, 2006). Interferon responses can be elicited either through the cytosolic dsRNA-activated protein kinase PKR, or the toll-like receptors 3 and 7, that recognize RNA on the cell surface or in endosomes. Certain nucleotide motifs such as 5'-UGUGU-3' or 5'-GUCCUUCAA-3' within siRNAs appear to be responsible for the induction of interferon and interleukin production by plasmacytoid dendritic cells. Thus, it is important to avoid such motifs in the shRNA design (Manjunath *et al.*, 2009). In addition, the risk of IFN response induction may increase when dsRNAs longer than 30 bp are introduced in

mammalian cells (Manche *et al.*, 1992; Liu *et al.*, 2007). In this regard, it has been reported that lhRNAs and e-shRNAs of more than 50 bp, harboring multiple C to U (or A to G) mutations within the sense strand, can specifically inhibit gene expression, without inducing type I interferon (IFN) pathway. These mutations introduce wobble pairings along the stem region of the hairpins, that change their thermodynamic properties, allowing them to evade recognition by dsRNA-binding proteins, such as PKR. In addition, the use of these mutations may have several other advantages over perfectly complementary hairpins. For instance, mismatches may prevent deletions during plasmid propagation in *E.coli*, by avoiding the formation of stable hairpin structures; facilitate sequencing of the hairpins; abrogate RNAi-mediated cleavage of the vector transcripts, triggered by siRNAs produced from the hairpins during the packaging reactions, and allow more efficient reverse transcription of the vector after transduction (Sano *et al.*, 2008).

In summary, although endogenous shRNA expression has tremendous potential, it also possesses several intrinsic risks, that could be dangerous. Therefore, the importance of carefully designing shRNA constructs, to optimize the dose and the exact sequence of siRNAs, cannot be overemphasized (Manjunath *et al.*, 2009).

4.10 Potential molecular targets for anti-HIV-1 RNAi-based therapeutics

A number of criteria have been proposed for developing genetic inhibitors of HIV-1 for human clinical trials.

Firstly, it is important to target sequences that are conserved among different virus strains, to reduce the chance of mutant escape (Zhou and Rossi, 2011).

Secondly, anti-HIV-1 therapeutics that block entry and replication before virus integration are considered the best inhibitors, in comparison with the ones inhibiting later steps of the viral replication cycle (Scherer and Rossi, 2011). Indeed, mathematical modeling has predicted that post-integration inhibitors lead to the persistence of cells carrying an integrated provirus, resulting in an accumulation of HIV-1 infected cells that could ultimately counteract their antiviral effect. By contrast, inhibitors that act before integration, even those with

lower potency, are predicted to exert a systemic antiviral effect with the expansion of transduced cells capable of resisting HIV-1 infection (Hoxie and June, 2012).

Furthermore, it may also be beneficial to select target sequences in the early spliced mRNAs encoding the early HIV-1 proteins Tat, Rev and Nef. Indeed, an early block of viral gene expression will seriously hamper the expression of the late structural proteins and virion assembly (Berkhout, 2009). In this context, small interfering RNAs designed to destroy the *tat*/*rev* transcripts were found to be highly effective in viral suppression (Sano *et al.*, 2008).

In addition, the many cellular factors that support HIV-1 replication cycle can be successfully targeted. This alternative RNAi strategy seems attractive because the genetic barrier for viral escape may be significantly higher. For instance, HIV-1 adaptation to another cellular co-factor may be impossible when no alternative cellular functions are available (Berkhout, 2009). While hundreds of human genes which depletion inhibited either p24 production or viral gene activities have been identified, the CCR5 co-receptor remains one of the most promising cellular target for anti-HIV-1 therapeutic approaches. Indeed, heterozygous or homozygous individuals for a 32-base-pair deletion in the CCR5 gene (CCR5Δ32), that prevents CCR5 expression on the cell surface, are slower progressors or resistant to HIV-1 infection, respectively (Samson *et al.*, 1996; Scherer and Rossi, 2011; Hütter and Ganepola, 2011a). Epidemiological studies of the Caucasian population demonstrated that the CCR5Δ32 deletion shows the highest frequency of 10-20% among the heterozygous and 1% among the homozygous karyotype. On the contrary, this deletion cannot be found in the Asian, Middle East, African and the American Indian population (Martinson *et al.*, 1997; Hütter and Ganepola, 2011b). The absence of any other significant phenotype associated with a lack of CCR5 has spurred the development of therapies aimed at blocking the virus-CCR5 interaction, and CCR5 antagonists have proved to be an effective salvage therapy in patients with drug-resistant strains of HIV-1 (Holt *et al.*, 2010). Importantly, Hütter and colleagues reported the case of a patient, known as the "Berlin patient", with acute myeloid leukemia, who was cured of AIDS following a bone marrow transplant from a donor homozygous for CCR5Δ32. The engrafted donor phenotype appears to have conferred long-term control of HIV-1 replication, as the patient has been off HAART for several years without HIV-1

being detected (Hütter *et al.*, 2009; Hütter and Ganepola 2011a; Allers *et al.*, 2011; Burke *et al.*, 2013). However, the identification of human leukocyte antigen-matched CCR5Δ32 homozygous donors for transplantation presents a significant logistical barrier to the general application of this approach (Li *et al.*, 2013). Therefore, various gene therapy approaches to block CCR5 expression are being evaluated, including both mature T cells and CD34+ HSCs as target cells. Of note, loss of CCR5 in HSCs appears to have no adverse effects on hematopoiesis (Holt *et al.*, 2010).

4.11 Biosafety of retroviral and lentiviral vectors as gene delivery systems

The first clinical trial using a lentiviral vector was initiated in 2003 and involved the transduction of CD4+ T cells from HIV-1-positive patients to express an antisense sequence targeting the HIV-1 *env* gene (Levine *et al.*, 2006). Lentiviral vectors are being successfully used with increasing frequency in human clinical trials (Aiuti *et al.*, 2013; Biffi *et al.*, 2013). Nonetheless, as the first retroviral vectors developed, MLV-based gammaretroviral vectors have had the longest-standing and broadest clinical use and have accounted for approximately 20% of gene therapy clinical trials worldwide as of January 2012 (McGarrity *et al.*, 2013; Naldini, 2011).

Lentiviral vectors have distinct characteristics that favor their use in delivery and long-term gene expression in human clinical trials. These include the ability to accommodate large gene inserts and to transduce both dividing and nondividing cells, the high levels and the prolonged duration of transgene expression (McGarrity *et al.*, 2013). On the contrary, gammaretroviral vectors are known to be prone to silencing of expression by DNA methilation, that specifically targets the LTR sequences. Moreover, for integration to occur, gammaretroviral vectors require cells to enter division shortly after infection, thus imposing the need for prolonged culture under conditions of active proliferation. This requirement may become a severe hurdle when *ex vivo* culture is detrimental to the maintenance of relevant stem cell properties and may cause differentiation, as it has long been the case with HSCs (Naldini *et al.*, 2011).

Insertional genotoxicity is another important issue to consider when using an integrating vector for gene therapy purposes. Indeed, insertions could give rise to dominant gain-of-function mutations, such as the activation of proto-oncogenes flanking an insertion site, or loss-of-function mutations in tumor suppressor genes. These events are more likely in gammaretroviral vectors, which have a bias for integration near promoters of active genes (Bushman *et al.*, 2005; Montini *et al.*, 2009; Sharma *et al.*, 2013). Several leukemia cases have been described in two SCID-X1 gene therapy trials that employed a gammaretroviral vector (Hacein-Bey-Abina *et al.*, 2003a,b; Gaspar *et al.*, 2011). In all these patients, the therapeutic vector integrated near proto-oncogenes and altered their expression, thereby promoting clonal T cell proliferation (Hacein-Bey-Abina *et al.*, 2008). As opposed to MLV, lentiviruses tend to integrate into intronic regions of genes, without any bias towards insertion near promoters (De Palma *et al.*, 2005; Montini *et al.*, 2006; Zhou et al., 2010; Cattoglio *et al.*, 2010; Cartier *et al.*, 2012). In addition, the natural history of HIV-1 shows that, despite an overwhelming viremia in HIV-1 infected patients, especially in the first weeks of infection, where approximately 1-10x10^9 virus particles are produced in patients per day, there is no single report of a lentivirus-induced tumor (McGarrity *et al.*, 2013). Montini and coworkers exploited HSCs from tumor-prone mice to assess the oncogenicity of prototypical gammaretroviral and lentiviral vectors (Montini *et al.*, 2006). Results provided evidence that gammaretroviral vectors triggered dose-dependent acceleration of tumor onset. Insertions at oncogenes and cell-cycle genes were enriched in early-onset tumors, indicating cooperation in tumorigenesis. By contrast, tumorigenesis was unaffected by lentiviral vectors and did not enrich for specific integrants, despite the higher integration load and robust expression of lentiviral vectors in all hematopoietic lineages. These data demonstrated that lentiviral vectors have low oncogenic potential, highlighting a major rationale for application to gene therapy (Montini *et al.*, 2006). The use of SIN LTRs in the last generation lentiviral vectors further reduces the chance of insertional genotoxicity, thanks to the deletion of enhancer and promoter elements comprised within the U3 region of the LTR (Naldini, 2011).

MLV-based retroviral vectors present an additional potential safety problem: the generation of replication-competent retroviruses during vector production. This

event may occur following recombination during reverse transcription between the vector RNA and passively incapsidated gag/pol and env RNA sequences within the vector particle. This is a point of particular concern when the vector stock is destined for a clinical trial. In contrast, and despite extensive production and thorough testing of HIV-1 derived vectors, the presence of replicative HIV-1 in lentivector stocks has never been described (reviewed by Di Nunzio *et al.*, 2012).

Although these evidences on the safety of lentiviral vectors are encouraging, quantitative analysis awaits the results of the safety monitoring of patients who have received lentiviral vectors in the setting of human clinical trials (McGarrity *et al.*, 2013).

5. AIM OF THE STUDY

The overall goal of this research project is to explore the use of genetic medicine to immunize hematopoietic stem cells (HSCs) harvested from HIV-1-positive patients against viral infection. In this context, patients affected by AIDS-related lymphoma (ARL) offer a unique opportunity to evaluate anti-HIV-1 gene therapy strategies in an ethically acceptable clinical setting, as they often undergo HSC transplantation. Once infused back into the patients, the genetically modified HSCs would build up an HIV-1-resistant lymphohematopoietic system that could lead to a significantly reduced viral load with stable and complete remission of the underlying lymphoid malignancy.

Starting from these considerations, this study is aimed to develop lentiviral vectors expressing multiple siRNAs interfering with the virus life cycle at different steps and to test their efficacy in human primary cells, including CD4+ T lymphocytes and macrophages. The siRNA targets we selected include the *CCR5* cellular gene and the *tat*, *rev* and *vif* viral genes. In order to optimize vector design, we compared the antiviral activity and the safety of alternative strategies used for multiple siRNA delivery and tested the activity of different promoters. This study would contribute to the identification of new anti-HIV-1 combinatorial platforms that, once shown to be effective and safe *in vivo*, may be next in line for clinical testing.

6. MATERIALS AND METHODS

6.1 Vector construction

The human U6 and 7SK polymerase III promoters were amplified from 293T and HeLa genomic DNA, respectively, flanked by *Eco*RI and *Mlu*I sites [U6 promoter primers: 5'-GAATTCAAGGTCGGGCAGGAAGAGGGCCTA-3' and 5'-ACGCGTGCACGGTGTTTCGTCCTTTCCACA-3' (GenBank: X07425.1); 7SK promoter primers: 5'-GAATTCCTGCAGTATTTAGCATGCCCCACC-3' and 5'-ACGCGTCCGAGGTACCCAGGCGGCGCACAA-3' (GenBank: X05490.1)]. The H1 RNA polymerase III promoter was derived from the pLVTHM vector (Wiznerowicz and Trono, 2003), digested with *Eco*RI and *Mlu*I.

To obtain the shCCR5, the shvif and the scrambled sequence, two complementary DNA oligonucleotides flanked by *Mlu*I and *Cla*I sites were annealed and cloned at *Eco*RV site of the pBluescript II KS plasmid (Stratagene). Sequences of the forward oligonucleotides were as follows: shCCR5 5'-GAGCAAGCTCAGTTTACACCTTCAAGAGAGGTGTAAACTGAGCTTGCTCTTTTT-3' (Liang *et al.*, 2010); shvif 5'-GTTCAGAAGTACACATCCCTTCAAGAGAGGGATGTGTACTTCTGAACTTTTT-3' (Lee *et al.*, 2005); scrambled 5'-GAGCAAGCTC**TCG**TTACACCTTCAAGAGAGGTGTAA**CGA**GAGCTTGCTCTTTTT-3' (Liang *et al.*, 2010). The shRNA sequences feature a loop situated between the sense and the reverse complementary sequences (underlined) and a polyT terminator at the 3' end. The scrambled control sequence contains three mismatched nucleotides as compared to the shCCR5 (bold).

To obtain the lhRNA and the e-shRNA sequences a two-step PCR approach was undertaken. The first PCR was carried out with the same forward primer employed to amplify the U6 promoter and a reverse primer specific for either the lhRNA or the e-shRNA. A plasmid containing the U6 promoter and 153 nt of the downstream snU6 RNA gene was used as a template. Sequences of the reverse primers were as follows: lhRNA 5'-TCTCTTGAAGAGAAACTTGATAAGTCTAACTGTTCTAATGAACTCTTCATCGCTATCTCCGCACGCGTAAACAGAAAAACAA-3'; e-shRNA 5'-

TCTCTTGAAGGGATGTATACTTCTAAACATACTCCACTTCTTCCTACCA
TGTGGGTATAAACTAAGCTTACTCACGCGTAAACAGAAAAACAA-3'.
These primers are complementary to the last 20 nt of the snU6 RNA gene and to the sense and the 9-nt loop of the respective hairpin. *Mlu*I site was inserted between the end of the snU6 gene and the first nucleotide of the hairpin, to facilitate subsequent cloning. PCR conditions included 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 30 cycles. One or 0.5 µl of this reaction were used as a template for a second PCR step with the U6 forward primer and the following reverse primers: lhRNA 5'-
ATCGATAAAAAGCGGAGACAGCGACGAAGAGCTCATCAGAACAGTCA
GACTCATCAAGCTTCTCTCTCTTGAA-3'; e-shRNA 5'-
ATCGATAAAAAGAGCAAGCTCAGTTTACACCCACATGGCAGGAAGAA
GCGGAGTATGTTCAGAAGTACACATCCCTCTCTTGAAA-3'. These primers harbour sequences complementary to the 9-nt loop appended to the antisense strand of the respective hairpin, the polymerase III terminator sequence and the *Cla*I site. PCR to obtain the lhRNA was carried out following the same conditions described above, while amplification to produce the e-shRNA was performed as follows: 40" at 98°C, 1 min at 55°C and 1 min at 72°C for 30 cycles. The PCR fragments [including the full-length sequence of either the lhRNA: 5'-
GCGGAGATAGCGATGAAGAGTTCATTAGAACAGTTAGACTTATCAAGT
TTCTCTTCAAGAGAGAGAAGCTTGATGAGTCTGACTGTTCTGATGAGC
TCTTCGTCGCTGTCTCCGCTTTTT-3' (Sano *et al.*, 2008), or the e-shRNA: 5'-
GAGTAAGCTTAGTTTATACCCACATGGTAGGAAGAAGTGGAGTATGTT
TAGAAGTATACATCCCTTCAAGAGAGGGATGTGTACTTCTGAACATAC
TCCGCTTCTTCCTGCCATGTGGGTGTAAACTGAGCTTGCTCTTTTT-3']
were directly cloned into the *Eco*RV site of the pBluescript II KS plasmid.
The Pol III promoter (i.e. U6, 7SK or H1) was inserted immediately upstream of the shRNA, lhRNA or e-shRNA into the pBluescript II KS plasmid.
The third-generation, replication-defective, SIN lentiviral vector pLentiLox3.7 (pLL3.7) was previously described by Rubinson *et al.* (2003) (Figure 6.1A). This vector contains a hybrid 5' LTR in which the U3 region is replaced with the cytomegalovirus (CMV) promoter and enhancer sequence, the packaging signal (Ψ), the RRE sequence, the flap sequence or central polypurine tract (cPPT), the

central termination sequence (CTS), the enhanced green fluorescent protein (EGFP) reporter gene driven by an internal CMV promoter, the woodchuck post-transcriptional regulatory element (WPRE) and the 3' LTR in which the *cis* regulatory sequences are completely removed from the U3 region. pLL3.7 backbone contains as well a murine U6 promoter between *Xba*I-*Xho*I sites. This vector is thereafter referred to as the empty vector.

To construct lentiviral vectors expressing one single hairpin molecule, the shRNA, lhRNA or e-shRNA transcriptional unit was subcloned into pLL3.7 between the *Xba*I and *Xho*I sites, in place of the murine U6 promoter.

Lentiviral vectors expressing three hairpin molecules were constructed starting from the pBluescript II KS plasmids containing one single hairpin cassette. Initially, the plasmid encoding the first cassette was *Cla*I digested and protruding ends were filled-in by the Klenow fragment of DNA polymerase. The linearized plasmid was then *Sal*I digested in order to allow the subsequent ligation to the second cassette, that was contained on a *Sma*I-*Sal*I fragment derived from a different pBluescript II KS plasmid. The obtained plasmid, encoding two out of the three hairpin cassettes, was linearized by *Sal*I digestion and cut with *Xho*I, after treatment with the Klenow enzyme. Next, the third cassette was inserted with the *Xho*I-*Sma*I restriction sites. Finally, the fragment containing the triple cassette was excised with *Xba*I-*Xho*I and inserted into the pLL3.7 backbone.

The resulting plasmids were confirmed by restriction enzyme digestion and DNA sequencing.

6.2 Cell cultures

Human embryonic kidney 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen).

The canine thymocyte line stably expressing the human CD4 and CCR5 receptors (Cf2Th CCR5+/CD4+) was propagated in DMEM supplemented with 10% FBS, G418 (0.5 mg/ml) (Gibco) and Zeocin (0.3 mg/ml) (Invitrogen).

Jurkat cells (Clone E6-1) and C8166 cells were maintained in Roswell Park Memorial Institute's 1640 medium (RPMI) (Invitrogen) supplemented with 10% FBS.

Human primary monocyte-derived macrophages (MDMs) were obtained from buffy coats of healthy blood donors by Ficoll-Paque PLUS (GE Healthcare) purification, followed by plastic adherence of human peripheral blood mononuclear cells (PBMCs) for 1 h in RPMI 10% FBS. Non-adherent cells were removed and adherent cells were washed with PBS and cultured in RPMI containing 10% FBS and macrophage colony-stimulating factor (M-CSF) (500 U/ml) (Miltenyi Biotec) for 7 days to differentiate into macrophages. Preparation purity was evaluated by measuring the percentage of CD14-positive cells through FACS analysis, as described below (6.10). The cut-off employed to accept the purity of MDM preparation was a CD14-positive percentage higher than 90%.

Human primary CD4+ T lymphocytes were isolated from 1:2 diluted buffy coats by Rosette Sep (StemCell Technologies), according to the manufacturer's instructions. This method is based on negative selection of CD4+ T cells, to isolate untouched cells without the risk of activating or damaging them. Briefly, an antibody cocktail was directly added to the blood sample to cross-link unwanted cells to red blood cells. The antibody complexes were, then, separated from the remaining cells by gradient centrifugation, using Ficoll-Paque PLUS. The purity of the CD4+ T cell population ranged from 95 to 100%, as estimated by FACS analysis using monoclonal antibodies against the human CD4, CD8, CD14 and CD19 antigens (6.10). CD4+ T lymphocytes were cultured in RPMI medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and phytohemagglutinin (PHA) (5%) (EuroClone). Two days later, PHA was removed and cells were used for vector transduction, before IL-2 stimulation (6.6). In order to compare yield and purity of different CD4+ T lymphocytes purification protocols, the T Cell Isolation Kit II (Miltenyi Biotec) was used for the negative selection of CD4+ T cells from PBMCs. To this end, PBMCs were obtained from buffy coats after Ficoll-Paque PLUS purification and CD4+ T lymphocytes were isolated according to the manufacturer's instructions. The purity of the CD4+ T cell population was assessed by FACS analysis (6.10).

6.3 Luciferase assay

The psiCheck2 reporter plasmids were prepared by directed insertion of the RNAi target sequence into the *Xho*I-*Not*I sites of the psiCheck2 plasmid (Promega), such that the target sequence was within the 3' UTR of the *Renilla* luciferase gene. To obtain RNAi target sequence, two complementary DNA oligonucleotides flanked by *Xho*I and *Not*I sites were annealed. Sequences of the forward oligonucleotide are indicated as follows: shCCR5 target 5'-CAAGAGGCTCCCGAGCGAGCAAGCTCAGTTTACACCCGATCCACTGGGGAGCA-3' (GenBank:X91492.1); shvif target 5'-CCCTCATCCAAGAATAAGTTCAGAAGTACACATCCCACTAGGGGATGCTAGATTG-3' (B.FR.83.HXB2); lhtat/rev target 5'-GCGGAGACAGCGACGAAGAGCTCATCAGAACAGTCAGACTCATCAAGCTTCTC-3' (B.FR.83.HXB2); e-shRNA-derived sitat/rev target 5'-CCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGAGCT-3'(B.FR.83.HXB2). The RNAi target sequence is underlined.

For the luciferase assays, 293T cells were plated 1 day before transfection in 96-well plates at a density of $1x10^4$ cells/well in 100 µl of DMEM 10% FBS. Cells were co-transfected with 50 ng of the psiCheck2-derived plasmid and 300 ng of the siRNA-expressing vector, using Lipofectamine 2000 reagent (Invitrogen) (0.5 µl in DMEM serum free to a total volume of 50 µl/well). When three different psiCheck2 reporter plasmids were simultaneously tested, 50 ng of each plasmid were used for the transfection. Cells were assayed for luciferase expression with the Dual Glo Luciferase Assay System (Promega) 48 h post-transfection, according to the manufacturer's instructions, using a Centro LB 960 luminometer (Berthold Technologies). Renilla luciferase expression was divided by background firefly luciferase expression. Average expression ratios for the control vector encoding the scrambled sequence was set to 100% and relative expression levels for the other samples calculated accordingly. Three independent experiments in triplicate were performed and the data are expressed as the mean ± standard deviation.

6.4 Lentiviral vector production

Recombinant lentiviral vectors were produced by calcium phosphate transfection of 293T cells. Briefly, 2.5×10^6 cells were seeded on 10 cm Petri dishes and, when subconfluent, they were co-transfected with 15 µg of the appropriate gene transfer vector, 5 µg of the pMDL plasmid (Dull *et al.*, 1998), 3 µg of the pCMV-Rev plasmid and 1,5 µg of the pCMV-G plasmid (Li *et al.*, 2003) (Figure 6.1). The culture medium was changed 6 h later. When necessary, the next day DMEM 10% FBS medium was replaced with RPMI 10% FBS. The culture supernatants were collected 48 h after transfection, passed through a 0.45-µm-pore-size filter (Millipore) and stored at -80°C until use. When required, the supernatants were concentrated by ultracentrifugation. The viral titer was measured by the reverse transcriptase activity assay (6.5) and the infectious titer was determined in 293T cells by transducing with serial dilutions of the lentiviral stocks in 6-well plates. After 72 h, the percentage of EGFP+ cells was determined by flow cytometry (6.10). Viral titers typically ranged from $5x10^7$ to $5x10^8$ transducing units (TU)/ml for the non-concentrated lentivector stocks and from $5x10^8$ to $2x10^9$ TU/ml for the concentrated ones.

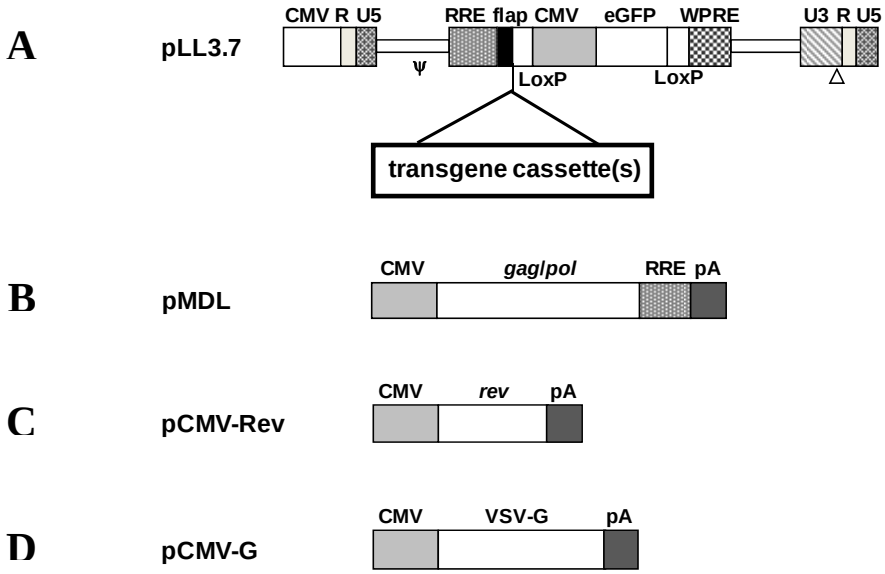


Figure 6.1. The lentiviral vector and the vector production system. (A) The transfer vector pLL3.7 contains a hybrid 5' LTR in which the U3 region is replaced by the CMV promoter, the packaging signal (Ψ), the RRE sequence, the flap sequence, the CMV-driven EGFP gene flanked by loxP sites, the WPRE sequence and the 3' LTR in which the *cis*-regulatory sequences have been completely removed from the U3 region. The gene(s) of interest (along with a human Pol III promoter) can be inserted upstream of the LoxP site which precedes the CMV-EGFP cassette in the vector. (B) pMDL contains the *gag* and *pol* genes and the RRE sequence from HIV-1 under the control of the CMV promoter. (C) pCMV-Rev contains the coding sequence of Rev driven by the CMV promoter. (D) pCMV-G contains the VSV-G protein gene under the control of the CMV promoter. pA indicates the polyadenylation signal from the human globin gene.

6.5 Reverse transcriptase (RT) activity assay

The RT activity was measured as previously described (Rho *et al.*, 1981). Briefly, viral particles were precipitated from 500 µl of the filtered culture supernatants by centrifugation at 13000 rpm for 2 h at 4°C. The precipitate was resuspended in 10 µl of a buffer containing 50 mM Tris-HCl pH 7.5, 1mM dithiothreitol (DTT), 20% glycerol, 250 mM KCl and 0.25% Triton X-100, transferred in dry ice and lysed through three cycles of freezing and thawing. The sample was added to a reaction mixture containing 50 mM Tris-HCl pH 7.5, 7.5 mM $MgCl_2$, 0.05% Triton X-100, 5 mM DTT, 100 µg/ml polyA, 10 µg/ml oligo-dT and 74 KBq of ^{3}H-dTTP (2.934 TBq/mmol) in a final volume of 50 µl. The reaction was incubated for 1 h at 37°C and transferred on Whatman filters. Filters were immediately washed three times in SSC 2X (0.3 M NaCl, 0.03 M sodium citrate pH 7.2) for 10 minutes each, twice in absolute ethanol for 10 seconds each and,

then, dried. The radioactivity was measured by using a scintillator (Rackbeta 1214 Wallac) and expressed in counts per minute (cpm).

6.6 Transduction of target cells

For transduction of 293T cells, $2.5x10^5$ cells were seeded per well in 6-well plates in 2 ml of DMEM 10% FBS. The next day, medium was replaced with 1 ml of medium containing equivalent amounts of vector particles (300000 cpm) and polybrene (8 μg/ml). Fresh culture medium was added to the cells approximately 8 h later. Two to three days after transduction, cells were detached from the tissue culture, with one aliquot used for FACS analysis of EGFP+ cells (6.10) and the remaining aliquots replated into 6-well plates at $2.5x10^5$ cells/well either for recombinant HIV-1 challenge (6.7) or Gag expression analysis (6.9).

Cf2Th CCR5+/CD4+ cells were plated at $2x10^5$ cells/well in 6-well plates, cultured overnight and transduced with lentiviral vectors at an m.o.i. of 50 TU/cell in 1 ml of DMEM 10% FBS without antibiotics, in the presence of polybrene (8 μg/ml). Approximately 8 h later, medium was removed and replaced with 2 ml of fresh DMEM 10% FBS containing G418 and Zeocin. Three days after transduction, the Cf2Th CCR5+/CD4+ target cells were detached from the culture plate by treatment with PBS and 5 mM EDTA. The cell suspension was diluted in medium and stained with monoclonal antibody to human CCR5 for FACS analysis (6.10).

Transduction of MDMs was performed over two consecutive days by incubating $1x10^6$ cells with vectors (from $1x10^7$ to $1x10^8$ TU in different experiments) in 1 ml of RPMI 10% FBS. After transduction, macrophages were maintained in culture medium supplemented with M-CSF (500 U/ml) for 72 h, before FACS analysis of CCR5 cell surface expression (6.10).

For transduction of Jurkat cells, $1x10^6$ cells were incubated with vectors at an m.o.i. of 50-100 TU/cell, in a total volume of 1 ml. After three days of culture, the transduction efficiency was ascertained by FACS analysis on the basis of EGFP expression (6.10) and cells were used for HIV-1 infection (6.7).

CD4+ T lymphocytes ($1x10^6$ cells) were incubated with the lentiviral vectors at an m.o.i. of 50 TU/cell in the presence of 8 μg/ml polybrene and spin-infected at

1200 rpm for 2 h at 25°C. After spin-inoculation, fresh RPMI 10% FBS medium containing IL-2 (100 U/ml) (R&D Systems), penicillin (100 U/ml) and streptomycin (100 µg/ml) was added to the cells. GFP, CD4 and CCR5 expression was analyzed by FACS at multiple time points after transduction (6.10). Of note, although an m.o.i. of 50 was used for transduction with different vectors, actual transduction efficiency appeared to vary from sample to sample, depending on the initial titers of the vector preparations. At day 4 after CD4+ T lymphocytes transduction, homogeneous EGFP+ populations were obtained by flow cytometric sorting and used for HIV-1 infection (6.7).

6.7 Wild-type and recombinant HIV-1 stock production and infection

HIV-1 HXBc2 Vpr+ Vpu+ Nef+ was generated by transfection of $5x10^6$ Jurkat cells with 10 µg of the pSVC Vpr+ Vpu+ Nef+ construct by the DEAE-dextran technique. This plasmid is a derivative of the pSVC21, containing the HIV-1 HXBc2 molecular clone (Ratner *et al.*, 1985), where the *vpr*, *vpu* and *nef* sequences were substituted with those derived from the pNL4-3 (*vpr*/*vpu*) (Adachi *et al.*, 1986) and pLAI (*nef*) (Peden *et al.*, 1991) molecular clones, in order to introduce functional *vpr*, *vpu* and *nef* genes. Jurkat cell supernatants were harvested at approximately 48 h post-trasfection and filtered (pore size, 0.45 µm). Viral titer was determined as $TCDI_{50}$ (50% tissue culture infectious dose)/ml on C8166 cells using the Reed and Muench method (Jawetz *et al.*, 1980), as well as by measuring the RT activity (6.5).

HIV-1 NL4-3-ADA stocks were produced by calcium phosphate transfection with 15 µg of the infectious proviral plasmid (gift from H. Göttlinger, University of Massachusetts Medical School) in 293T cells, as described above (6.4). pNL4-3-ADA plasmid is a derivative of the pNL4-3, containing the HIV-1 NL4-3 molecular clone, where the *env* sequence was replaced with that derived from the CCR5-tropic ADA strain (Theodore *et al.*, 1996). RT assay was performed in order to determine viral titer (6.5).

Four days after vector transduction (6.6), $1x10^6$ Jurkat cells were infected with HXBc2 Vpr+ Vpu+ Nef+ at an m.o.i. of 0.1 infectious units (IU)/cell for 1 h at 37°C, in a total volume of 300 µl. After the incubation, the cells were washed

three times with PBS, seeded in 12-well plates at a density of $1x10^6$ cells/ml and cultured in RPMI 10% FBS medium. The culture supernatants were collected at different days post-infection and the RT activity was measured (6.5).

For CD4+ T lymphocytes infection, $1x10^6$ activated EGFP+ (6.6) or control cells (untransduced CD4+ T cells) were challenged with equivalent reverse transcriptase units (10000 cpm) of either HXBc2 Vpr+ Vpu+ Nef+ or NL4-3-ADA at 24 h after cell sorting. Infection was carried out as previously described and cells were plated with RPMI 10% FBS supplemented with penicillin (100 U/ml), streptomycin (100 g/ml) and IL-2 (100 U/ml). The RT activity in culture supernatants was monitored at different time points after infection (6.5).

Recombinant HIV-1 virus to be used for 293T infection was produced by co-transfection of 293T cells with 5 µg of pHXBΔenvCAT and 3 µg of pCMV-G, by the calcium phosphate method (6.4). The pHXBΔenvCAT plasmid contains the HXBc2 HIV-1 provirus with a 580 bp deletion in the *env* gene and the chloramphenicol acetyltransferase (CAT) gene replacing the *nef* gene (Terwilliger *et al.*, 1989; Helseth *et al.*, 1990). All the recombinant HIV-1 viruses were quantified by RT assay (6.5).

Transduced 293T cells (6.6) were infected by incubation with the recombinant virus (10000 cpm) in 1 ml of medium. After overnight incubation at 37°C, fresh medium was added to the cells and, two days later, they were lysed and used for determination of CAT activity (6.8).

6.8 Chloramphenicol acetyltransferase (CAT) activity assay

For CAT activity assays, cells were lysed in 150 µl of 250 mM Tris-HCl pH 7.5 and protein concentration in the lysates was determined with the BCA Protein Assay Kit (Thermo Scientific) using BSA as a standard, according to the manufacturer's instructions. Equivalent amounts of proteins were used for determination of CAT activity, as previously described (Sodroski *et al.*, 1984). The different forms of acetylated chloramphenicol were separated by thin layer chromatography (TLC) and visualized with an autoradiografic exposure of 12 h (Kodak Biomax films). The quantitative evaluation was obtained by cutting the TLC paper at the level of the corresponding spots, and by performing a

quantification of the spots at the scintillator. The percentage of conversion in the acetylated forms was calculated as follows: % of conversion = (mono- + di-acetylated forms)/(non acetylated + mono-. + di-acetylated forms). Calculated with the above formula, the percentage of conversion is linear for values up to 50%.

6.9 Gag expression analysis

Transduced 293T cells (6.6) or control cells were transfected by the calcium phosphate technique with pCMV-Rev, pMDL, or with both plasmids (6.4; Figure 6.1B-C). At 24 h cells were harvested, washed with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer [140 mM NaCl, 8mM Na_2HPO_4, 2 mM NaH_2PO_4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulphate (SDS)]. For immunoblot analysis, proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto a Protran membrane (Whatman). The membranes were incubated with the appropriate primary antibody, namely a rabbit polyclonal anti-HIV-1 capsid antiserum (anti-HIV-1 p24 Gag antiserum; ABi Advanced Biotechnologies) or a mouse anti-α-tubulin antibody (Monoclonal Anti-α-Tubulin antibody, Sigma). Blots were visualized with a peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (GE Healthcare) and developed with enhanced chemiluminescence reagents (GE Healthcare), as described elsewhere (Strack *et al.*, 2000).

6.10 FACS analysis

Cells (typically $5x10^5$) were stained with monoclonal antibodies to human CCR5 (APC Mouse Anti-Human CD195, BD Pharmingen), CD4 (PE-Cy7 Mouse Anti-Human CD4, BD Pharmingen), CD14 (CD14-PE, human, Miltenyi Biotec), CD8 (Anti-Human CD8a APC, eBioscience) or CD19 (CD19-FITC, human, Miltenyi Biotec), according to the manufacturer's instructions. The cells were also stained with isotype controls for each of the specific antibodies, as well as with the 7-Amino-Actinomycin D (7-AAD) viability dye (BD Pharmingen). When necessary, cells were either incubated with FcR blocking reagent (Miltenyi

Biotec) before staining or fixed with 2% formaldehyde before acquisition on the flow cytometer. Samples were acquired on either a LSRII or FACSCalibur (Becton Dickinson) and the data analysis was performed with FlowJo (Tree Star) or CellQuest (Becton Dickinson) software, respectively.

6.11 MTT cell viability assay

293T cells were seeded at a density of $1x10^4$ cells/well in 100 µl of DMEM 10% FBS in 96-well plates and grown one day before the transfection with lentiviral vectors (300 ng), using Lipofectamine 2000 reagent, as previously described (6.3). At 48 h post-transfection, MTT (Roche) was added according to the manufacturer's instructions.

Jurkat cells and CD4+ T lymphocytes were plated in 96-well plates at a density of 10^5 cells/well in 100 µl of RPMI 10% FBS, 72 h post-transduction (6.6). After overnight recovery, MTT assay was performed.

Optical density was measured at 620 nm and the value obtained for control cells (untransfected/untransduced cells) was set to 100%. Relative cell viability for other samples was calculated accordingly. Three independent experiments in triplicate were performed and the data are expressed as the mean ± standard deviation.

6.12 Cloning and analysis of lentiviral vector insertion sites

Vector integration sites were determined by linker-mediated polymerase chain reaction (LM-PCR) followed by Sanger sequencing of amplicons, as previously described (Cattoglio *et al.*, 2007). Briefly, genomic DNA was extracted from 0.5 to $5x10^6$ transduced CD4+ T lymphocytes (6.6), digested with *Mse*I and ligated to a TA-protruding linker, obtained by annealing the following oligonucleotides: 5'-GTAATACGACTCACTATAGGGCTCCGCTTAAGGGAC-3' and 5'-TAGTCCCTTAAGCGGAG-3'. The genomic DNA was further digested with *Sac*I/*Nar*I, in order to prevent amplification of internal vector sequences. Next, LM-PCR was performed with primers annealing to the vector 3′ LTR and the linker (3' LTR primer: 5′-AGTGCTTCAAGTAGTGTGTGCC-3′; linker primer:

5’-GTAATACGACTCACTATAGGGC-3’; 3’ LTR nested primer: 5′-CGTATCGCCTCCCTCGCGCCATCAGCGTAGTCTGTTGTGTGACTCTGGTAAC-3′; linker nested primer: 5’-CTATGCGCCTTGCCAGCCCGCTCAGAGGGCTCCGCTTAAGGGAC-3’).

LM-PCR libraries were subjected to gel-size selection in a range of 250-700 bp. Amplicons were gel-purified (Gel-Extraction Kit, Qiagen) and shotgun-cloned (TOPO TA Cloning Kit, Invitrogen) into DH5α *E.coli* cells. Finally, LM-PCR amplicons were Sanger-sequenced, generating a collection of vector-human genome junction reads.

Valid reads consisted of the following sequences: the 3’ LTR nested primer, the last part of the vector LTR up to a CA dinucleotide, the adjacent human genome up to the first *Mse*I site and the linker nested primer. Human genome sequences longer than 20 bp were mapped onto the hg19 release of the human genome by the University of California Santa Cruz (UCSC) BLAT alignment tool (http://genome.ucsc.edu) and not univocally mapping sequences were discarted. The genomic coordinates of the first nucleotide following the LTR were considered as the vector integration site (IS). Only sequences featuring a unique best hit with at least 95% of identity to the human genome were taken into account to annotate *bona fide* ISs.

ISs were classified as inside or outside genes when occurring within or outside a gene-body region (RefSeq genes collection, hg19 release). Exon or intron localization of ISs were determined by manually-curated inspection on the UCSC Genome Browser tool (http://genome.ucsc.edu).

Functional annotation of ISs targeted genes was performed by the DAVID 6.7 Functional Annotation tool (http://david.abcc.ncifcrf.gov). Functional categories are derived from the Gene Ontology (GO)-Biological Process classification. Statistical significance of any given category with respect to the human genome, used as background population, was set at a p-value < 0.05.

7. RESULTS

7.1 Selection of anti-HIV-1 shRNAs and development of lentiviral vectors expressing the specific shRNAs as single transcriptional units

To inhibit HIV-1 infection at multiple stages of the virus life cycle, an original combination of three RNAi agents, which individually confer resistance to viral infection, was tested. Their targets include the transcripts of the CCR5 cellular gene and those of the *vif* and the *tat/rev* viral genes.

CCR5 is mainly expressed on the surface of CD4+ T lymphocytes and monocyte/macrophages and it is used by most HIV-1 strains as a co-receptor to enter target cells. Several evidences make CCR5 an intriguing target for HIV-1 therapy. Firstly, individuals homozygous for the truncated Δ32 variant of the CCR5 gene are resistant to HIV-1 infection and are otherwise healthy (Samson *et al.*, 1996). Secondly, targeting of cellular co-factors that are essential for HIV-1 replication represents a promising anti-escape approach, since the mutation rate of the cellular DNA replication machinery is significantly lower than that of the lentiviral reverse transcriptase enzyme. Thus, the chance that resistance mutations are selected in host mRNAs is negligible compared to HIV-1 target sequences (Knoepfel *et al.*, 2012). Thirdly, anti-HIV-1 genes which block pre-integration steps of HIV-1 infection will prevent the generation of provirus and viral reservoirs, that are the main reasons for the failure to cure HIV-infected individuals (Walker *et al.*, 2012). Taken together, these considerations prompted us to select a shRNA targeting CCR5 (shCCR5), that has been previously described by Chen and co-workers (Liang *et al.*, 2010). This molecule has been shown to have antiviral potency both in HSCs and in non-human primates, in the absence of cytotoxicity (Liang *et al.*, 2010; An *et al.*, 2007).

Vif is a viral accessory protein that enhances virus infectivity and is essential for viral replication and pathogenesis *in vivo*. Vif counteracts the restriction factors of the cellular APOBEC3 cytosine deaminases family (i.e. APOBEC3G and APOBEC3F) by inducing their proteasomal degradation. In the absence of Vif expression, the APOBEC3 proteins are incorporated into the newly synthesized virus particles, leading to the hypermutation in the viral DNA during reverse

transcription in the next round of infection (Mangeat *et al.*, 2003). In addition, it has been demonstrated that APOBEC3G also induces defects in reverse transcription and DNA integration (Bishop *et al.*, 2008; Mbisa *et al.*, 2007). We selected a shRNA targeting a highly conserved sequence within the *vif* open reading frame (shvif) (Lee *et al.*, 2005), that has been proven to be effective against viruses from multiple clades, including primary viral isolates from clades A, B, C, D and E.

Tat and Rev are regulatory viral gene products, essential for viral gene expression. The former transactivates transcription from the HIV-1 LTR by binding to the TAR element at the 5' end of all viral mRNAs, while the latter promotes the export of unspliced or singly spliced viral mRNAs from the nucleus by binding to the RRE sequence (Feng and Holland, 1988; Malim *et al.*, 1989). Interestingly, the *tat* and *rev* first exons overlap, allowing to interfere with both gene transcripts by means of a single siRNA. To inhibit Tat and Rev expression, we selected a long hairpin RNA (lhRNA) generating two distinct siRNAs against contiguous sequences in the *tat*/*rev* common transcript (lhtat/rev) (Sano *et al.*, 2008). In this context, mutants that arise should have a selective disadvantage because the reading frames for the Tat and Rev proteins are different. Therefore, silent third-position codon changes for one gene will very often result in an amino acid alteration for the other gene, thereby affecting expression of that gene. G:U wobble parings were included in the sense strand of the lhtat/rev, to attenuate the innate immune response to long dsRNAs.

In general, the shRNA/lhRNA design was based on the prototype shRNA hairpin transcript published by Brummelkamp and coworkers in 2002: complementary sense and antisense strands, a 9-nucleotide hairpin loop and 3'-UU overhang (Brummelkamp *et al.*, 2002). The antisense strand of this shRNA design, upon Dicer processing, will form the guide strand that instructs RISC for antiviral attack. The complete shRNA/lhRNA cassette consists of a human RNA polymerase III promoter and the shRNA/lhRNA sequence followed by the TTTTT termination signal (Figure 7.1A-B). The selected promoters included the human U6 small nuclear RNA promoter (U6) and the human RNase P RNA H1 promoter (H1), which represent the two most commonly used promoters to drive shRNA expression. In addition, the 7SK small nuclear RNA promoter (7SK) was

further adopted, to better characterize its activity when used to express RNAi triggers. Promoters were either amplified from human genomic DNA or obtained as a restriction fragment from the appropriate plasmid. shCCR5 and shvif were constructed as synthetic DNA, while the lhtat/rev was generated by a two-step PCR approach, as described in details in the Material and Methods section (6.1). Initially, the single shRNA/lhRNA cassette was cloned in a prototypical 3rd generation SIN lentiviral vector (Rubinson *et al.*, 2003). As indicated in Figure 7.1C, a total of nine different vectors encoding the shRNA cassette in the forward orientation were constructed, to define the best combination of promoter-RNAi effector. An additional vector expressing the H1-driven lhtat/rev unit in the antisense orientation was obtained during the cloning steps, and turned out to be useful in order to assess the influence of promoter orientation on siRNA efficacy. Moreover, three scrambled vectors to be used as controls were developed. Each of these expresses a shRNA, under the control of either the U6, the 7SK or the H1 promoter, generating an siRNA that is three nucleotides different from the one produced by the shCCR5. All the vectors harbor the CMV-driven EGFP reporter gene, as a marker for tracking transduced cells.

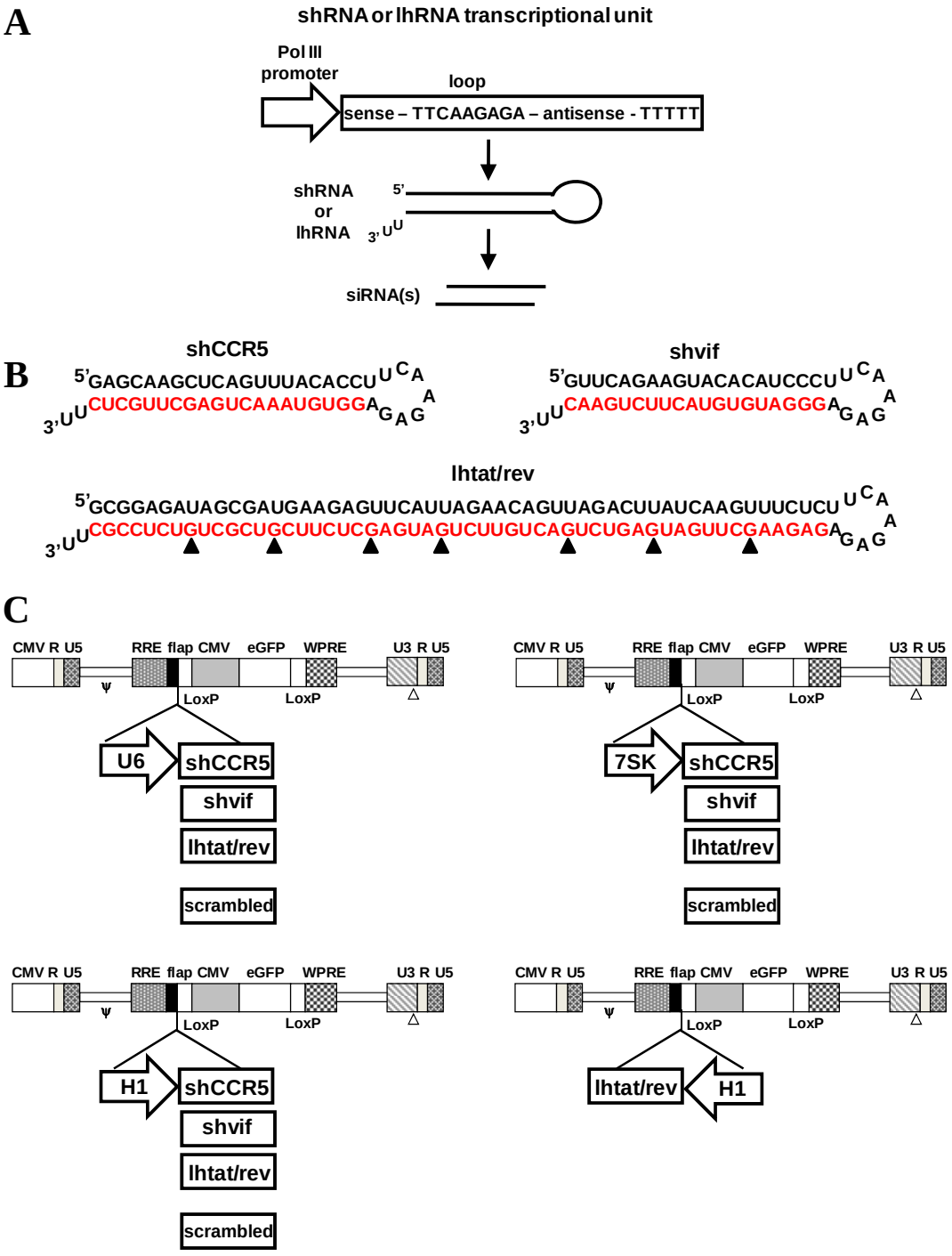
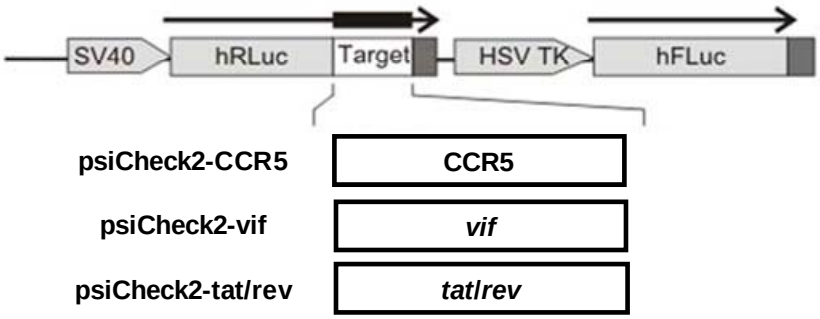


Figure 7.1. Schematic diagram of the single shRNA-expressing vectors. (A) Schematic illustration of a Pol III promoter-driven shRNA or lhRNA transcriptional unit. A sequence of 2 U nucleotides that are derived from the transcription termination signal is shown. The intended mechanism of transcription and processing of the hairpin molecule to form one or multiple siRNAs is illustrated. (B) Sequence and predicted structure of the shRNAs and lhRNA. The guide strand is marked in red, while G:U pairings are indicated with an arrowhead. (C) A self-inactivating third generation lentiviral vector, pLL3.7, was utilized to derive the anti-HIV-1 constructs. The single shRNA or lhRNA along with its independent Pol III promoter is inserted upstream of the LoxP site, which precedes the EGFP transcriptional unit. Arrows indicate the transcriptional orientation of the RNAi trigger cassette.

7.2 Activity of the single shRNA vectors by means of the luciferase knockdown assay

Initially, to evaluate the silencing activity of the single shRNA-expressing vectors, 293T cells were co-transfected with each vector along with a reporter plasmid encoding the *renilla* luciferase gene fused with the respective RNAi target sequence. In addition, the reporter plasmid encodes the *firefly* luciferase gene to control for transfection efficiency (psiCheck2-CCR5, psiCheck2-vif, psiCheck2-tat/rev) (Figure 7.2A). Renilla and firefly luciferase expression was measured 2 days after transfection and the ratio was used to calculate the relative luciferase activity. The renilla/firefly ratio in the presence of the scrambled vectors was set at 100%.

A



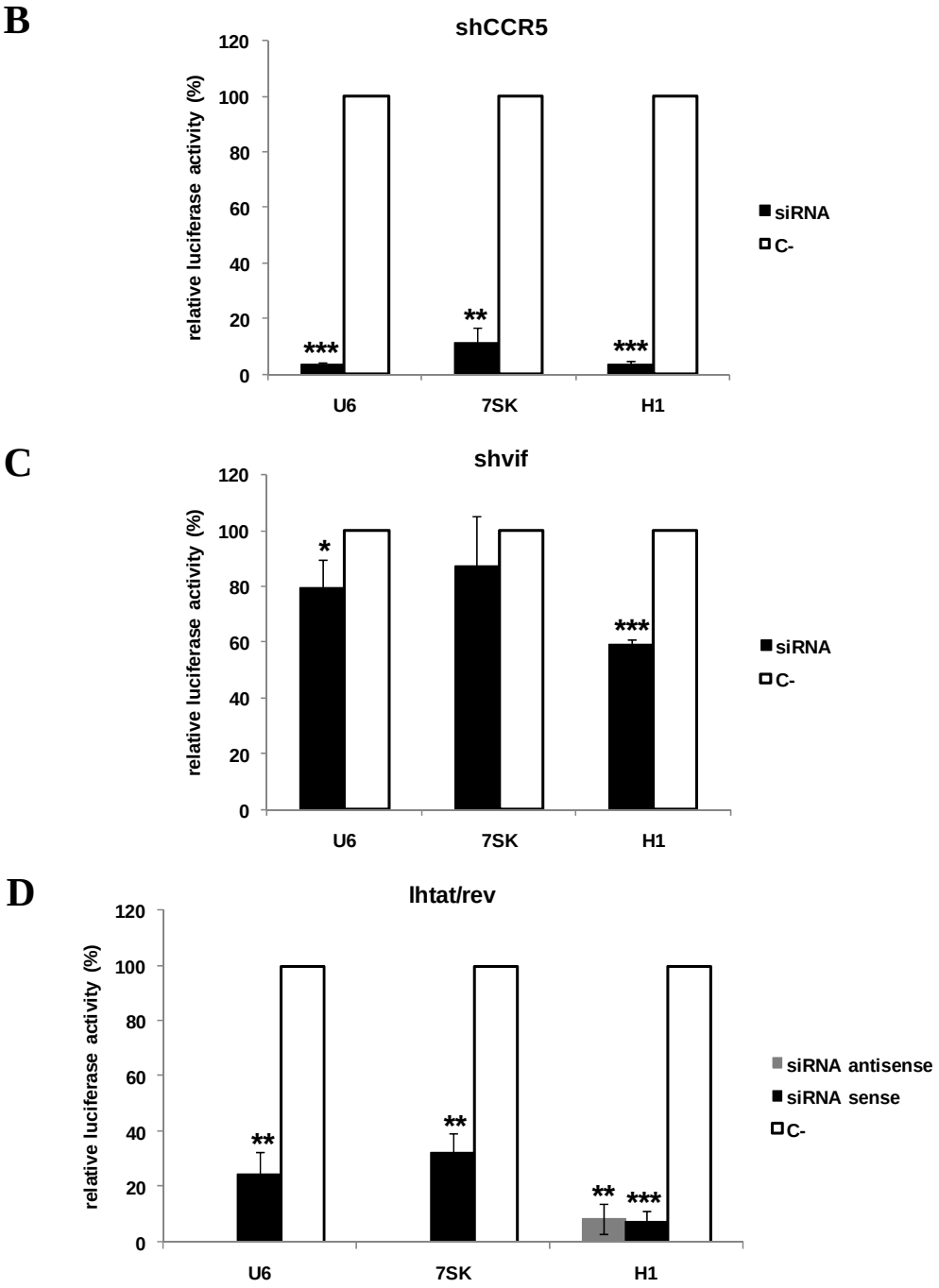


Figure 7.2. Knockdown of luciferase reporter by the single shRNA-expressing vectors. (A) psiCheck2-derived vectors that include the indicated RNAi target sequences inserted downstream of the *renilla* luciferase ORF. The control firefly luciferase cassette, present on the same plasmid, is also shown. *Renilla* and *firefly* luciferase genes are under the control of the simian virus 40 (SV40) and the herpes simplex virus thymidine kinase (HSV TK) promoter, respectively. (B-D) Average normalized ratios of the renilla:firefly luciferase activity when cells were transfected with the psiCheck2-CCR5, psiCheck2-vif or psiCheck2-tat/rev reporter plasmid along with the vectors expressing, respectively, the shCCR5 (B), shvif (C) or sense/antisense lhtat/rev (D), under the control of the indicated promoters. The scrambled hairpin has been included as a control (C-). The average values from three independent experiments, with standard deviations, are given (*, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; t-test, relative to C-).

When testing the shCCR5 vectors, highly effective knockdown of the renilla luciferase expression was achieved, with the U6 and the H1 promoters mediating an almost complete suppression of the reporter gene activity (Figure 7.2B). When the *vif* sequence was inserted downstream of the *renilla* luciferase gene, the shvif vectors mediated a less pronounced silencing activity. Indeed, only the H1-driven shvif was capable of 40% inhibition of reporter gene expression (Figure 7.2C). On the contrary, the lhtat/rev expression cassette diminished renilla luciferase activity by approximately 70-90%. In this context, the H1 promoter turned out to be the most efficient regardless of the cassette orientation (Figure 7.2D).

Taken together, these results indicated that siRNA activity is influenced by the used promoter, while promoter orientation does not appear to have a major impact on silencing efficacy. Thus, only vectors harboring the shRNA cassette in the forward orientation were used for subsequent experiments.

7.3 The shCCR5 downregulates CCR5 expression in cell cultures

To examine the activity of the shCCR5 in targeting the full length CCR5 transcript, the suppression of CCR5 cell surface expression was tested in canine Cf2Th thymocytes stably expressing the human CD4 and CCR5 molecules (Cf2Th CD4+/CCR5+) (LaBonte *et al.*, 2003). To this end, cells were transduced with vectors encoding either the shCCR5 or the scrambled hairpin at an m.o.i. of 50 TU/cell and, 72 h post-transduction, samples were analyzed by FACS for cell surface CCR5 expression. The results of a representative experiment performed with the H1-driven shCCR5 vector are reported in Figure 7.3.

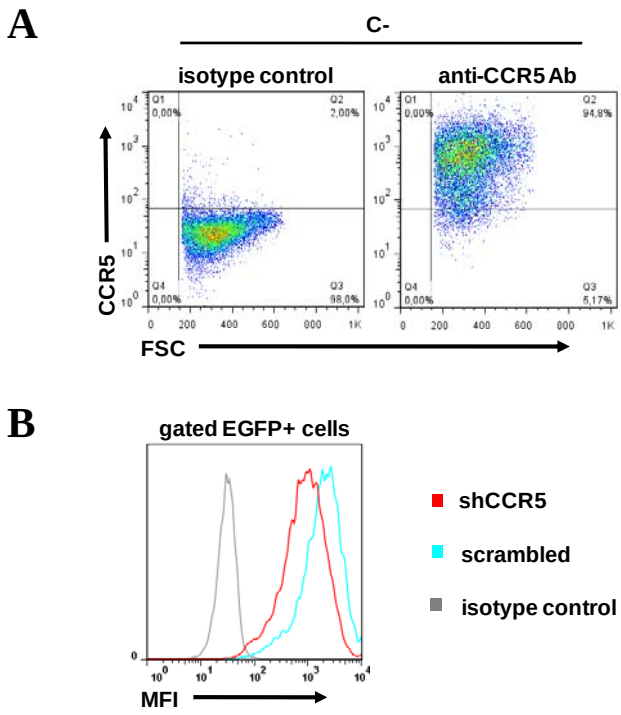


Figure 7.3. Reduction of CCR5 surface expression on CCR5+ Cf2Th canine thymocytes transduced with the shCCR5 vectors. Canine thymocytes expressing human CD4 and CCR5 were transduced with either the shCCR5-expressing vectors or the scrambled counterparts. The cells were harvested 3 days after transduction and analyzed by FACS with anti-human CCR5 or isotype control antibody staining. (A) CCR5 surface expression on control cells (C-). The results are exhibited as CCR5 vs forward scatter (FSC) dotplots with cell populations in the live canine thymocytes gate (typically > 90%). (B) FACS histogram showing the mean fluorescence intensity (MFI) for CCR5 in cells transduced with either the H1-shCCR5 vector (red curve) or the respective scrambled control (blue curve). The graph is referred to productively transduced cells, gated on the basis of their EGFP signal (EGFP+ cells).

Among the productively transduced thymocytes, i.e. 80% and 73% of EGFP+ cells for the shCCR5 vector and the scrambled vector, respectively (data not shown), CCR5 expression in shCCR5 population dropped to 35% with respect to the level of the scrambled-transduced cells (Figure 7.3B). Importantly, this result was observed despite the very high amount of CCR5 expressed on thymocytes surface, as compared to the physiological expression level on human primary macrophages.

To further investigate the activity of the shCCR5 in physiologically relevant cells, the suppression of CCR5 cell surface expression was assessed in human primary macrophages, which are the natural target of HIV-1 infection. As a first step, monocytes were purified from buffy coats of healthy blood donors by Ficoll-

Hystopaque gradient and cells were cultured in the presence of M-CSF to induce macrophage differentiation. The preparation purity was evaluated by measuring the percentage of CD14-positive cells through FACS analysis, considering a cut-off value higher than 90%. Next, transduction of monocyte-derived macrophages (MDMs) with vectors expressing the shCCR5 was performed over 2 consecutive days. As a control, macrophages were transduced with the scrambled vectors. CCR5 and EGFP expression level was assessed by FACS at approximately 72 hours after the second transduction. Transduction efficiency typically ranged from 20% to 60%, based on EGFP expression. Figure 7.4 shows FACS plots and trsnduction efficiency from two representative donors (1 and 2).

A

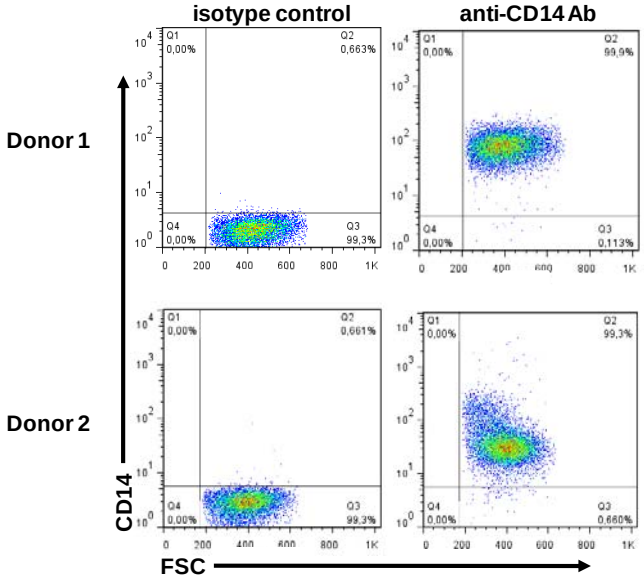


Table 1

Vector	EGFP+ cells (%)	
	Donor 1	Donor 2
U6shCCR5	47	26
U6scrambled	44	33
7SKshCCR5	39	28
7SKscrambled	28	29
H1shCCR5	23	33
H1scrambled	42	63

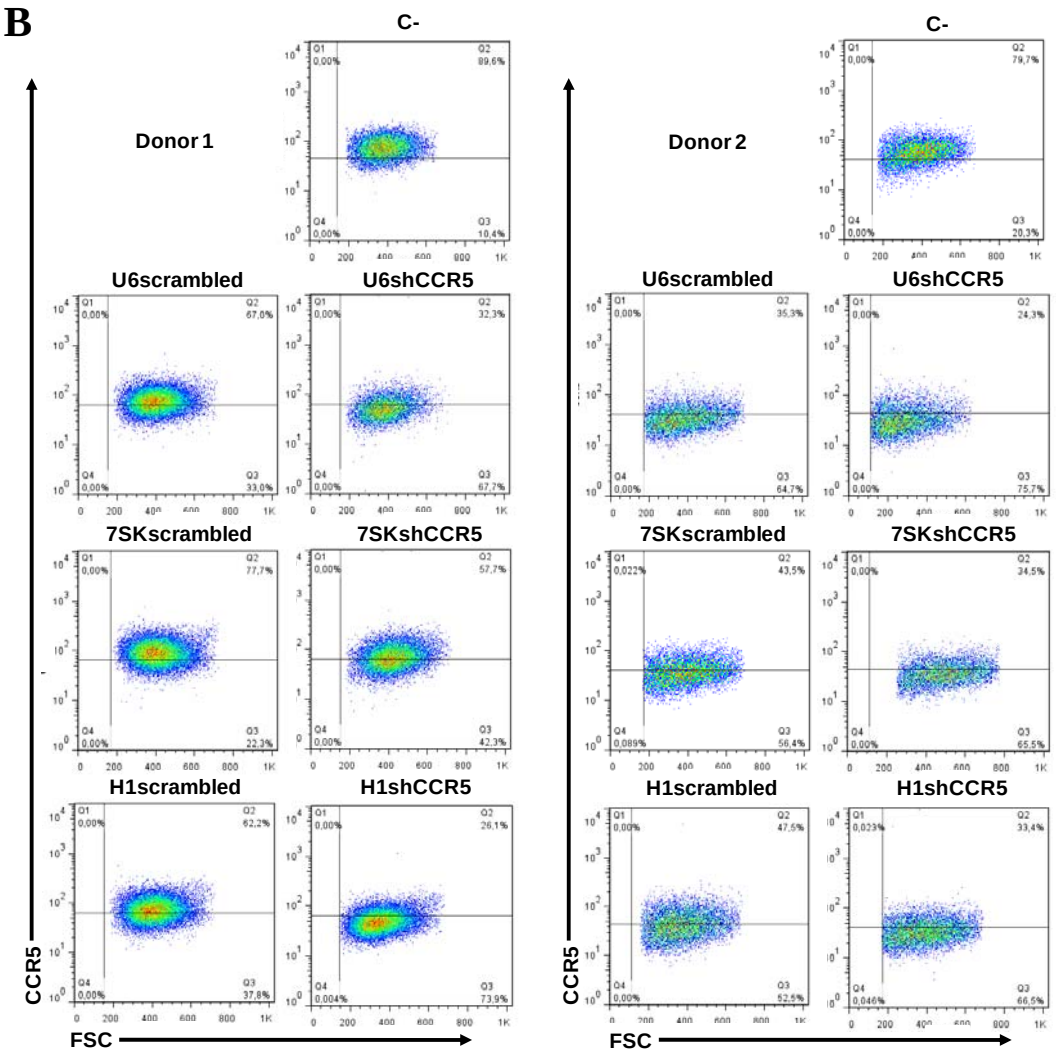


Figure 7.4. Reduction of CCR5 surface expression on human primary macrophages transduced with the shCCR5 vectors. (A) Monocytes derived from PBMCs were cultured in the presence of M-CSF for 7 days to differentiate to macrophages. MDM preparation purity was assessed by FACS analysis for CD14 expression on cell surface. (B) MDMs were transduced with vectors expressing the shCCR5 or the scrambled hairpin over 2 consecutive days. Cells were harvested 3 days after the second transduction and stained with anti-human CCR5 antibody. The expression of EGFP (Table 1) and CCR5 was analyzed by flow cytometry. Results from two donors are shown (1 and 2). The panel displays CCR5 vs FSC dotplots with cell populations in the live macrophage gate (typically > 40%). The quadrant lines were defined by isotype control staining.

As shown in Figure 7.4B (Donor 1) ≅ 90% of the control cells were CCR5+. A marked downregulation of CCR5 expression was observed when cells were transduced with the shCCR5 vectors in comparison with the respective scrambled controls. Specifically, the U6- and the H1-driven shCCR5 resulted in the most striking CCR5 reduction, according to the results obtained with the luciferase

assay (7.2). These data were confirmed with donor 2-derived macrophages. Of note, in this latter case an overall less pronounced CCR5 dowregulation by the shCCR5 was detected, suggesting a certain degree of inter-donor variability (Figure 7.4B, Donor 2).

7.4 Effect of the lhtat/rev on HIV-1 gene expression

Taking into account that the lhtat/rev produces two siRNAs, simultaneously targeting the HIV-1 *tat* and the *rev* genes, we sought to evaluate the anti-Tat and the anti-Rev effect independently of each other. With this aim, two different experimental setups were designed, as described below. Importantly, although the lhtat/rev target sites are also present in the Rev-expressing plasmid used to produce vector particles, there were no deleterious consequences on vector titers, as they were comparable to those obtained with the shCCR5, shvif and scrambled constructs.

7.4.1 Inhibition of Tat-dependent gene expression

In an effort to investigate the effect of targeting the *tat* viral transcript independently of the Rev downregulation, lhtat/rev-transduced 293T cells were challenged with an HIV-1 recombinant virus. To this end, a VSV-G-pseudotyped HIV-1 encoding the bacterial chloramphenicol acetyltransferase (CAT) gene in place of the *nef* gene was adopted. Since the recombinant virus contains a deletion in the *env* gene, it is capable of only one cycle of replication (Terwilliger *et al.*, 1989; Helseth *et al.*, 1990). CAT activity was, then, evaluated in the lysates of target cells. Indeed, in this context, the CAT gene transcription is Tat-dependent, as it is driven by the viral LTR, while the CAT transcript doesn't require Rev function, since it undergoes the same multiple splicing events used for the natural HIV-1 *nef* message.

293T cells were transduced with equivalent RT units (300000 cpm) of the lhtat/rev vectors or the scrambled counterpart. Once determined transduction efficiency on the basis of EGFP expression, cells were challenged with the CAT-reporter virus (10000 cpm) at 48 h post-transduction. Two days later, the

efficiency of CAT gene expression was evaluated in the target cells. The results of a representative experiment, that was performed two times, are reported in Figure 7.5.

Table 2

Vector	EGFP+ cells (%)
U6lhtat/rev	32
U6scrambled	42
7SKlhtat/rev	42
7SKscrambled	47
H1lhtat/rev	47
H1scrambled	29

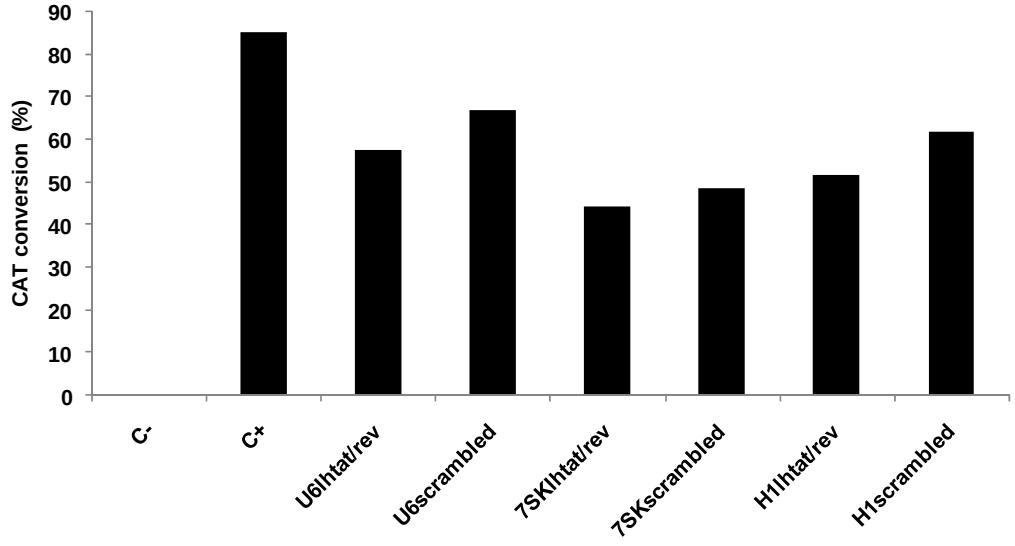


Figure 7.5. Effect of the lhtat/rev on Tat-dependent gene expression in 293T cells. 293T cells were transduced with either the lhtat/rev or the scrambled vectors and EGFP expression was analyzed by FACS 3 days post-transduction. The percentage of EGFP+ cells is reported (Table 2). Untransduced (C+) and transduced cells were exposed to a VSV-G pseudotyped HIV-1 reporter virus, encoding the CAT gene in place of the *nef* gene. The results of the CAT assay performed on the cell lysates two days post-infection are presented. One representative experiment is shown (C-: untransduced and uninfected cells).

Figure 7.5 highlights that vector-transduced cells exhibited an overall reduction of the CAT activity if compared with control cells (p-value < 0.001). However, when comparing the lhtat/rev-expressing cells with the respective scrambled counterpart, only a slight reduction in the CAT activity was detected. Considering that the transduced population that underwent viral challenge consisted of a mixture of EGFP-positive and EGFP-negative cells, we hypothesised that the effect of the lhtat/rev might have been partially masked by the EGFP-negative cells.

7.4.2 Inhibition of Rev-dependent gene expression

To dissect the role of Rev downregulation independently of Tat silencing effects, the expression of the HIV-1 *gag* gene under the control of a Tat-independent promoter was analyzed in 293T cells. To this end, lhtat/rev-transduced cells were co-transfected with a plasmid harboring the *gag* gene and the RRE sequence along with a plasmid encoding the Rev protein. In the presence of Rev, the *gag* transcript is exported from the nucleus to the cytoplasm, thanks to the binding of Rev to the RRE element, where it can be properly translated into the Gag precursor protein. On the contrary, in the absence of Rev expression, the *gag* transcript is retained into the nucleus and undergoes degradation. Importantly, as both Gag and Rev are expressed from a CMV promoter, it is possible to specifically evaluate the effect of lhRNA-mediated Rev downregulation.

As a first step, it was important to determine the minimum amount of the Rev-encoding plasmid required to express Gag. Thus, 293T cells were co-transfected with increasing amounts of the Rev plasmid along with a fixed amount of the Gag plasmid and harvested 24 h later. Proteins derived from the cell lysates were analyzed by SDS-PAGE, followed by Western blotting with an anti-HIV-1 Gag monoclonal antibody to examine Gag expression. It was found that a tiny amount of the Rev plasmid is sufficient to promote Gag expression, as demonstrated by the presence of both the Gag precursor protein (Pr55) and the mature capsid protein (p24) in cells transfected with as low as 10 ng of the Rev plasmid (Figure 7.6A).

Based on these results, the amount of the Rev plasmid used for transfecting lhtat/rev-expressing cells was accordingly decreased. Before transfection, transduced cells were FACS analyzed to assess transduction efficiency, that ranged from 30% to 50% for a typical experiment (data not shown). Next, transfection using only 1 ng of the Rev-expressing plasmid along with the Gag construct was carried out at 72 h post-transduction. Twenty-four hours later, cells were harvested and Gag expression was analyzed. As indicated in Figure 7.6B, the lhtat/rev caused a reduction in p24 protein expression as compared to the scrambled controls, regardless of the used promoter.

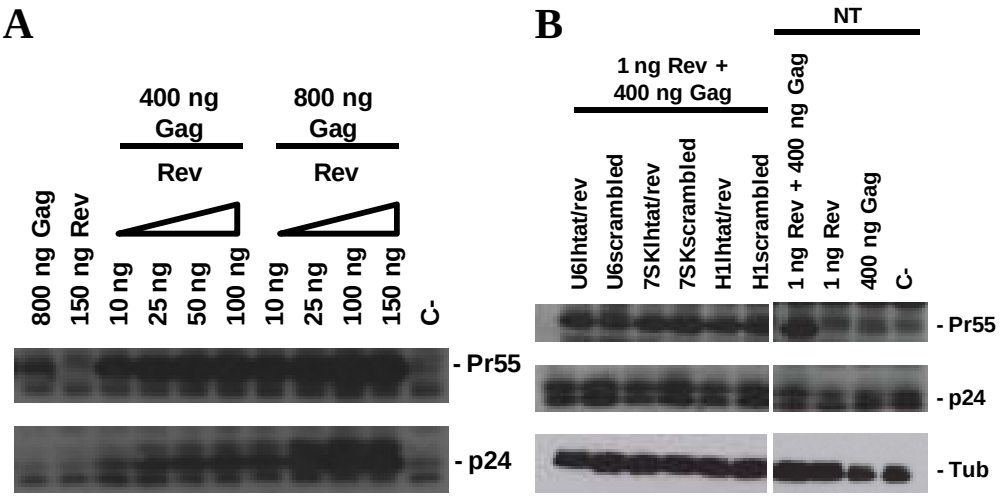


Figure 7.6. Effect of the lhtat/rev on Rev-dependent gene expression in 293T cells. (A) The HIV-1 Gag- or Rev-expressing constructs were transfected into 293T cells either alone (800 ng and 150 ng, respectively) or in combination, by using a fixed amount of the Gag-expressing plasmid (400 ng or 800 ng) along with increasing amounts of the Rev-expressing plasmid (10 ng to 150 ng), as indicated. At 24 h, the cells were lysed and proteins derived from the cell lysates were analyzed by SDS-PAGE, followed by Western blotting with an anti-HIV-1 Gag monoclonal antibody (Pr55 and p24) (C-: untransfected cells). (B) 293T cells, either untransduced (NT) or transduced with the lhtat/rev or the scrambled vectors, were transfected with the Gag- and Rev-expressing constructs (1 ng and 400 ng, respectively). As controls, NT cells were transfected with the Gag- or Rev-expressing plasmid alone, or neither of them (C-). Cell lysates were analyzed 24 h after transfection by SDS-PAGE, followed by Western blotting employing an anti-HIV-1 Gag monoclonal antibody (Pr55 and p24) or an anti-α-tubulin monoclonal antibody (Tub), as indicated.

7.5 Development of lentiviral vectors expressing a combination of anti-HIV-1 siRNAs

Similar to current antiviral drugs used in the clinic, the application of a single RNAi agent against HIV-1 is not sufficient to maintain inhibition. HIV-1 can escape from inhibition by mutating either its RNAi target sequence or a region outside the target sequence, that alter the local RNA secondary structure. Therefore, a successful RNAi-based therapy against HIV-1 requires the use of multiple siRNAs (ter Brake *et al.*, 2006). In this context, the simultaneous expression of multiple shRNA transcriptional units represents one of the most promising combinatorial strategies.

Starting from these considerations, we cloned the shCCR5, the shvif and the lhtat/rev in a single vector as independent transcriptional units. A number of vectors were constructed, differing from each other by either the promoter driving the expression of each shRNA or the position of the shRNA cassette (Figure 7.7A). One vector was obtained by combining each shRNA with the most efficient promoter, as resulted with the luciferase knockdown assay (i.e. U6shCCR5-H1lhtat/rev-H1shvif) (7.2). As a consequence, the vector contains two copies of the H1 promoter. Considering that the presence of repeated regulatory sequences has been associated with genetic instability and reduced titer of the vector system (Liu *et al.*, 2007; ter Brake *et al.*, 2008), different promoters were used for developing the other combinatorial vectors. Specifically, in another vector the U6 and the H1 promoters were maintained to express the shCCR5 and the lhtat/rev, respectively, which represent the most potent RNAi effectors, while the 7SK promoter was used to express the shvif (i.e. U6shCCR5-H1lhtat/rev-7SKshvif). In another combination, the H1lhtat/rev and the 7SKshvif cassettes were swapped, as compared to the above-described vector, in order to assess the impact of cassette position on vector antiviral activity (i.e. U6shCCR5-7SKshvif-H1lhtat/rev). Finally, in another context, the combination of promoter-shRNA was designed to optimize the overall silencing effect mediated by the three RNAi effectors (i.e. 7SKshCCR5-U6lhtat/rev-H1shvif).

In addition to the multiple shRNA cassette strategy, the use of extended shRNAs (e-shRNAs) has been shown to provide durable anti-HIV-1 inhibition (Liu *et al.*,

2009). e-shRNAs can express up to three siRNAs, stacked on top of each other along the stem of the hairpin, under the control of a single promoter.

To compare the efficacy of different combinatorial anti-HIV-1 platforms, we designed an e-shRNA encoding three siRNAs targeting the CCR5, the *tat*/*rev* and the *vif* transcripts, under the control of the U6, the 7SK or the H1 promoter (Figure 7.7B). The siRNAs against the CCR5 and the *vif* transcripts have the same target sequence as compared to the shCCR5 and the shvif, respectively. On the contrary, the siRNA against the *tat* and *rev* genes has a different target sequence with respect to both the siRNAs produced by the lhtat/rev. Indeed, since the stem region of the extended hairpin has an upper size limit of 66 bp for effective production of multiple and functional siRNAs (Liu *et al.*, 2009), the lhtat/rev guide strand sequence was replaced with a shorter one, generating one single siRNA that targets a distinct region of the *tat*/*rev* common transcript (Liu *et al.*, 2009). There is some evidence suggesting that Dicer processing of e-shRNAs may not be equal across the span of the duplex, favoring the production of the siRNAs generated from the hairpin stem base (Saayman *et al.*, 2008; Liu *et al.*, 2009). Thus, the potent siCCR5 was positioned as first, close to the stem base, followed by the sitat/rev and the sivif, ordered as second and third, respectively. This configuration allows the siRNA that blocks the very early stages of HIV-1 infection to be potentially expressed at higher levels than the ones interfering with post-integration steps. A three-nucleotide linker sequence was inserted between contiguous siRNAs, as this is the most frequently adopted spacer, even if only few studies addressed this point up to date (Liu *et al.*, 2007; Saayman *et al.*, 2008). Finally, G:U wobble base pairs were included at regular intervals in the sense strand of the e-shRNA, to suppress the innate immune response to dsRNAs and to facilitate the propagation of the e-shRNA-encoding plasmids in *E.coli* (Sano *et al.*, 2008; Saayman *et al.*, 2008).

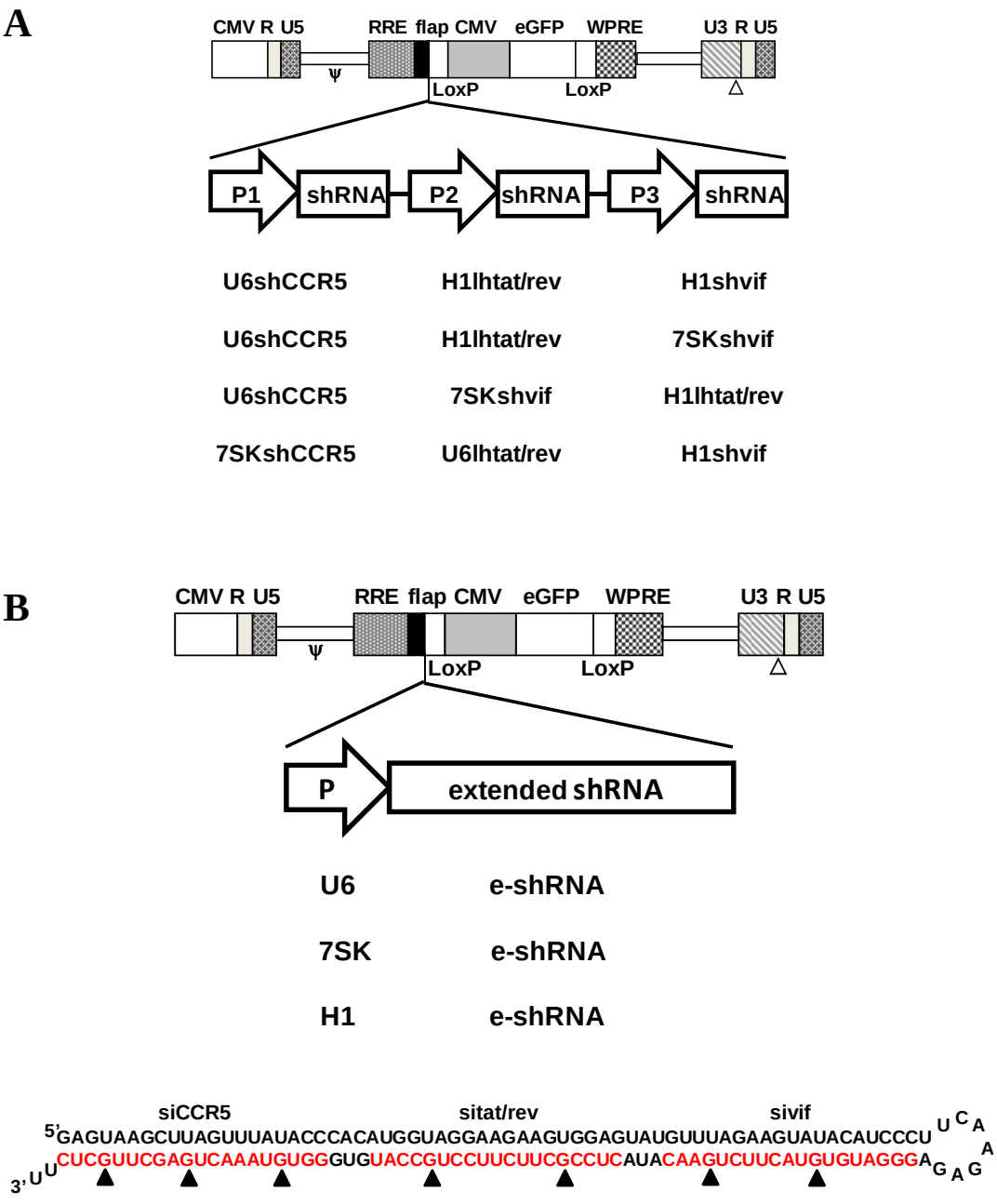
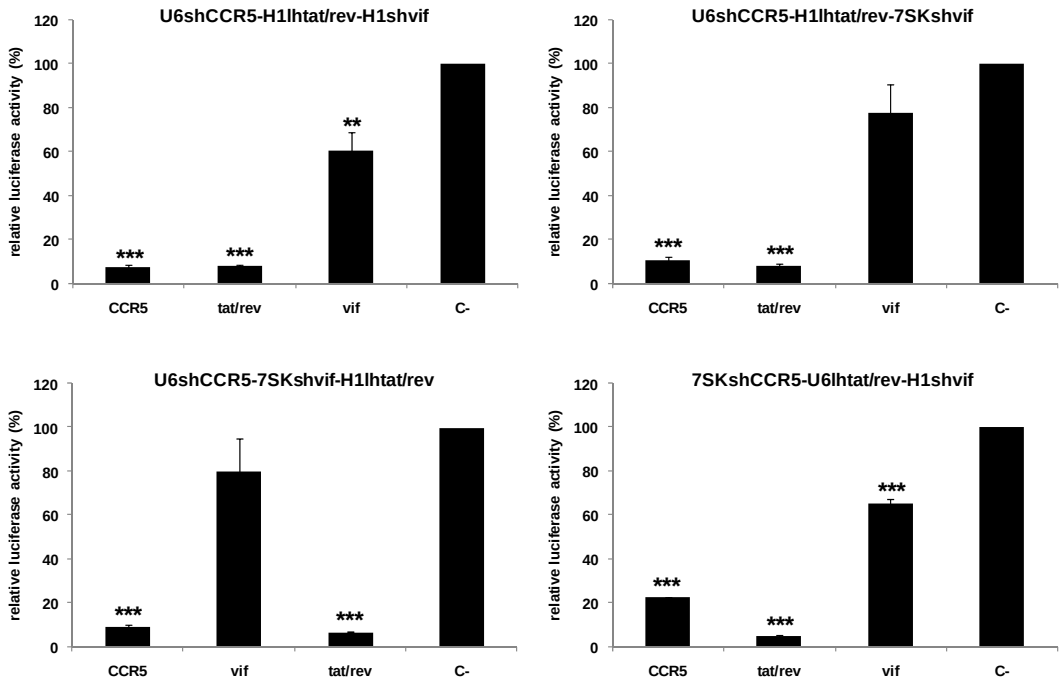


Figure 7.7. Schematic diagram of the combinatorial vectors. (A) The triple vectors contain a triple combination of anti-HIV-1 shRNAs and lhRNA expressed as independent transcriptional units. A total of four different triple vectors were obtained by using different promoters to express each RNAi trigger or by swapping the position of the RNAi trigger cassettes. Sequences and structure of the shRNAs and of the lhRNA have been previously described (Figure 7.1B). (B) The e-shRNA vectors encode a single hairpin encompassing 64 bp in the stem under the control of the U6, the 7SK or the H1 promoter. The e-shRNA gives rise to three distinct siRNAs targeting the CCR5, the *tat*/*rev* and the *vif* transcripts. The sequence and the predicted structure of the e-shRNA as well as the order of the siRNA-encoding sequences along the extent of the duplex are indicated. The guide strand sequences are marked in red, while G:U pairings are highlighted with an arrowhead (P: promoter).

7.6 Activity of the combinatorial vectors by means of the luciferase knockdown assay

To determine whether the simultaneous expression of multiple RNAi triggers could affect the activity of the single siRNAs, the luciferase assay was performed. Initially, 293T cells were co-transfected with each combinatorial vector along with one single luciferase reporter plasmid. As regards the triple shRNA vectors, the reporter plasmids were the same used for testing the single shRNA vectors (i.e. psiCheck2-CCR5, psiCheck2-vif, psiCheck2-tat/rev) (7.2). By contrast, as far as the e-shRNA vectors is concerned, a new reporter plasmid was obtained to assess the silencing activity of the sitat/rev, whose target sequence is different from the ones of the lhtat/rev (psiCheck2-tat/rev*).

A



B

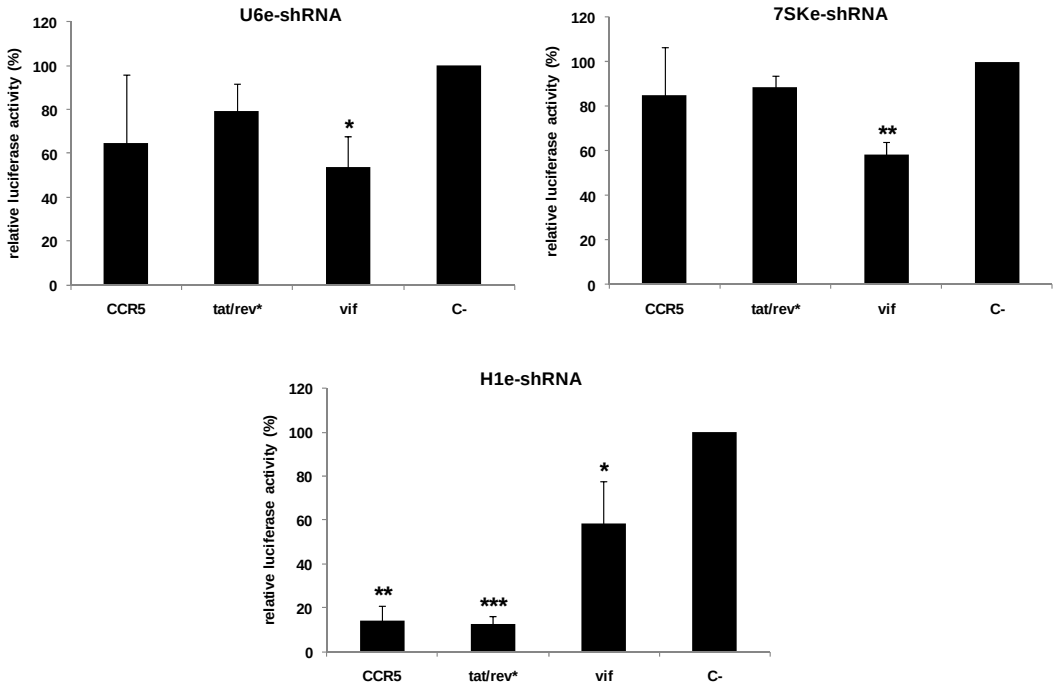


Figure 7.8. Knockdown of single luciferase reporter by the combinatorial vectors. Average normalized ratios of the renilla:firefly luciferase activity when cells were transfected with the indicated triple cassette (A) or e-shRNA (B) vector along with the psiCheck2-CCR5 (CCR5), psiCheck2-vif (vif), psiCheck2-tat/rev (tat/rev), or psiCheck2-tat/rev* (tat/rev*) reporter plasmid. As a control, cells were transfected with the corresponding reporter plasmid along with the vector(s) expressing the scrambled hairpin under the control of the U6, 7SK and/or H1 promoter, depending on the promoter driving the siRNAs. For convenience, only one control for each vector was plotted (C-). The average values from three independent experiments, with standard deviations, are given (*, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; t-test, relative to C-).

As shown in Figure 7.8A, the shCCR5 and the lhtat/rev maintained a highly effective silencing activity when expressed in the context of a triple shRNA vector. On the other hand, the shvif mediated a less pronounced reporter gene knockdown, confirming the results obtained with the single shvif vectors. This finding was consistent regardless of either the employed promoter or the cassette position. Altogether, these data demonstrated that multiple siRNAs can be efficiently expressed as independent transcriptional units from a single construct, resulting in similar levels of inhibition per shRNA compared to the corresponding single shRNA vector.

On the contrary, as regards the e-shRNA, the silencing activity of the three RNAi effectors was comparable to that of the respective single shRNA vectors only when the extended molecule was expressed from the H1 promoter. Remarkably, this was true also for the sivif, despite it was positioned close to the hairpin loop. By contrast, either the U6 or the 7SK promoter mediated a highly inefficient effect (Figure 7.8B).

Next, we moved further by co-transfecting reporters for CCR5, vif and tat/rev sequences all together along with each combinatorial vector. Indeed, this experimental setup allowed us to evaluate the overall silencing potential of the vectors, resembling the physiological condition of an HIV-1-infected cell expressing multiple siRNAs.

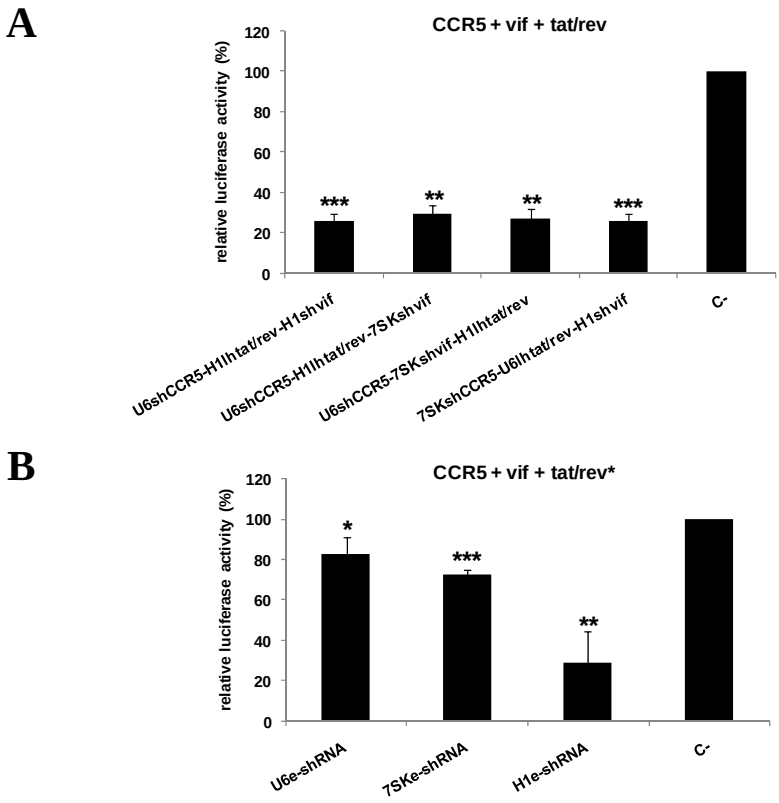


Figure 7.9. Knockdown of multiple luciferase reporters by the combinatorial vectors. Average normalized ratios of the renilla:firefly luciferase activity when cells were transfected with the indicated triple cassette (A) or e-shRNA (B) vector along with the psiCheck2-CCR5, psiCheck2-vif and psiCheck2-tat/rev or psiCheck2-tat/rev* reporter plasmids (CCR5 + vif + tat/rev or CCR5 + vif + tat/rev*). As a control, cells were transfected with all the reporter plasmids along with the vector(s) expressing the scrambled hairpin under the control of the U6, 7SK and/or H1 promoter, depending on the promoter driving the siRNAs. For convenience, only one control for each vector was plotted (C-). The average values from three independent experiments, with standard deviations, are given (*, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; t-test, relative to C-).

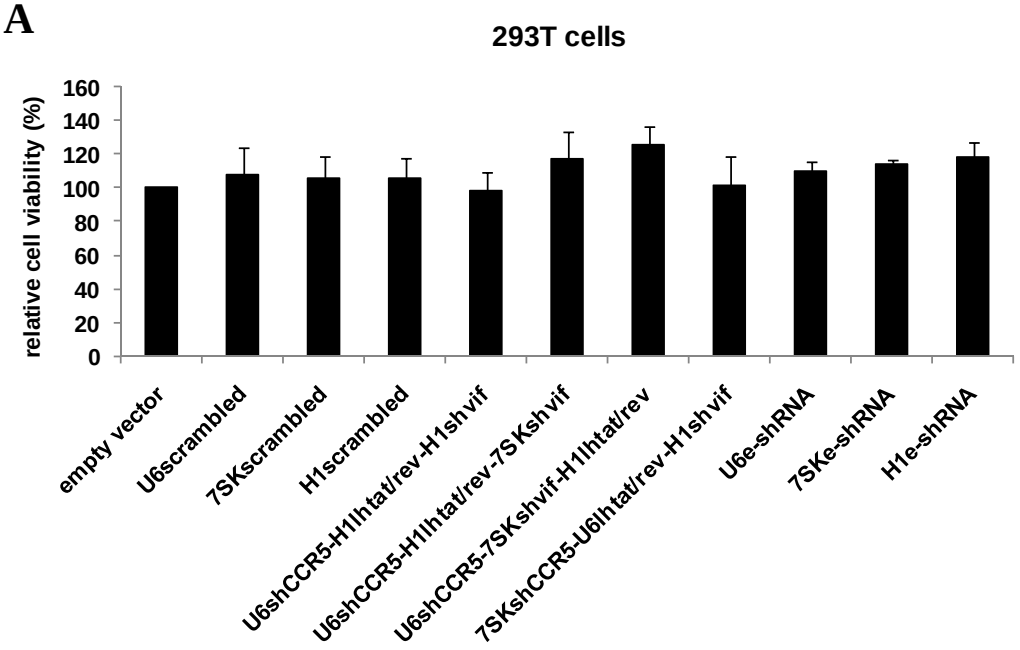
The triple shRNA vectors displayed an overall reporter gene suppression higher than 70%, without major differences among them (Figure 7.9A). The same extent of luciferase knockdown was achieved by the H1-driven e-shRNA, while both the U6- and the 7SK-driven e-shRNA triggered a considerably lower effect (Figure 7.9B). These results are consistent with data obtained by transfecting each reporter plasmid alone. Indeed, luciferase activity resulting from multiple reporter transfection was similar to the average activity calculated for the single reporter transfections, with respect to a given vector.

Taken together, these data suggested that all the developed triple vectors as well as the H1-e-shRNA vector can produce multiple active siRNAs that are equally efficient in knocking down their respective targets, upon transfection of target cells.

7.7 Analysis of combinatorial vector-related cytotoxicity

The expression of antiviral genes may induce undesired effects, which could compromise host cell function. High levels of siRNA expression could decrease cell viability and might activate the interferon (IFN) response pathway, which can cause attenuated cell growth and apoptosis (Ringpis *et al.*, 2012). To evaluate the biosafety of cells expressing multiple exogenous siRNAs, cell viability was assessed in cell cultures with and without siRNA expression. To this end, the MTT assay was undertaken in 293T cells, typically used for the production of lentiviral vector stocks, and in T lymphoblastoid Jurkat cells, a commonly used CD4+ T cell line permissive for HIV-1 infection. 293T cells underwent cell viability assay 48 h after transfection with either the combinatorial vectors or the scrambled counterpart, while Jurkat cells were assayed 72 h after vector transduction at an m.o.i. of 100 TU/cell. In all these experiments, the empty vector expressing no siRNAs was used as a control.

A


293T cells
relative cell viability (%)
160
140
120
100
80
60
40
20
0
empty vector
U6scrambled
7SKscrambled
H1scrambled
U6shCCR5-H1lhtat/rev-H1shvif
U6shCCR5-H1lhtat/rev-7SKshvif
U6shCCR5-7SKshvif-H1lhtat/rev
7SKshCCR5-U6lhtat/rev-H1shvif
U6e-shRNA
7SKe-shRNA
H1e-shRNA

B

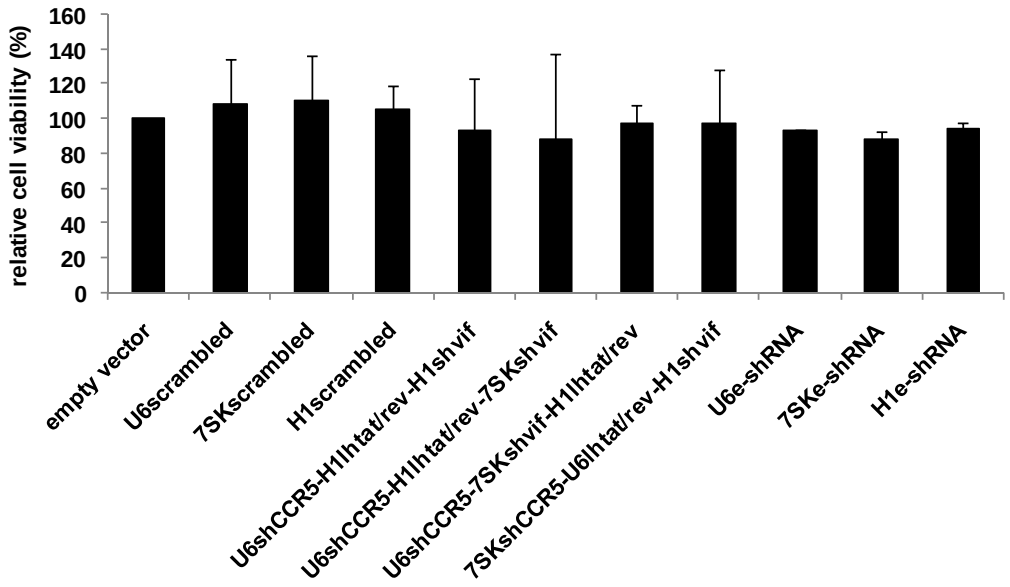

Jurkat cells
relative cell viability (%)
160
140
120
100
80
60
40
20
0
empty vector
U6scrambled
7SKscrambled
H1scrambled
U6shCCR5-H1lhtat/rev-H1shvif
U6shCCR5-H1lhtat/rev-7SKshvif
U6shCCR5-7SKshvif-H1lhtat/rev
7SKshCCR5-U6lhtat/rev-H1shvif
U6e-shRNA
7SKe-shRNA
H1e-shRNA

Table 3

Vector	EGFP+ cells (%)
empty vector	91 ± 12
U6scrambled	99 ± 1
7SKscrambled	95 ± 6
H1scrambled	99 ± 1
U6shCCR5-H1lhtat/rev-H1shvif	58 ± 9
U6shCCR5-H1lhtat/rev-7SKshvif	90 ± 14
U6shCCR5- 7SKshvif- H1lhtat/rev	100
7SKshCCR5-U6lhtat/rev-H1shvif	57 ± 3
U6e-shRNA	67 ± 3
7SKe-shRNA	67 ± 4
H1e-shRNA	63 ± 7

Figure 7.10. Absence of combinatorial vector-related cytotoxicity in cell lines. The pLL3.7 empty vector, the multiple siRNA-expressing vectors and the scrambled vectors were employed either to transfect 293T cells (A) or to transduce Jurkat T cells (B). At 2 or 3 days, respectively, cell viability was measured by MTT assay. Cell viability of either transfected or transduced cells was calculated by measuring the absorbance at 620 nm and normalizing it to empty vector-transfected or -transduced cells, respectively. The percentage of EGFP+ Jurkat cells 3 days after transduction is reported in Table 3. The means and standard deviations are derived from three independent experiments.

Results in Figure 7.10 indicated that cell viability remained unaffected in both 293T and Jurkat cells upon expression of multiple siRNAs. This was true despite the relatively high m.o.i. used for transduction of the latter cell line. FACS analysis revealed that transduction efficiency in Jurkat cells ranged between 57 and 100%, based on the EGFP expression (Table 3). Importantly, these data allowed us to rule out that the lack of efficacy of the U6 and 7SKe-shRNA vectors was due to a decreased cell viability.

7.8 Effect of the combinatorial vectors on HIV-1 replication in cell cultures

We next sought to evaluate the antiviral activity of the combinatorial vectors after *de novo* HIV-1 challenge. To this end, Jurkat cells were transduced with either each combinatorial vector (i.e. triple shRNA vectors and e-shRNA vectors) or control vector (i.e. U6-scrambled vector and empty vector), at an m.o.i. of 100 TU/cell, in order to achieve high efficiency of transduction, without cytotoxic effects (7.7). Three days after transduction, EGFP+ cells typically ranged from 67% to 97% (Table 4 and 5). Four days post-transduction, cells were challenged with the laboratory-adapted HXBc2 Vpr+ Vpu+ Nef+ strain of HIV-1. This virus encodes all the viral accessory proteins, resembling the scenario of an *in vivo* infection triggered by an HIV-1 primary isolate. Importantly, considering that the HXBc2 HIV-1 strain uses the CXCR4 co-receptor to enter target cells, in this experimental setup the contribution of the siCCR5 to the antiviral activity is not appreciable. Viral inhibition was assessed by measuring the RT activity in the cell culture supernatants at different time points.

Table 4

Vector	EGFP+ cells (%)
empty vector	90 ± 9
scrambled	96 ± 5
U6shCCR5-H1lhtat/rev-H1shvif	70 ± 22
U6shCCR5-H1lhtat/rev-7SKshvif	90 ± 10
U6shCCR5-7SKshvif-H1lhtat/rev	97 ± 5
7SKshCCR5-U6lhtat/rev-H1shvif	67 ± 17

A

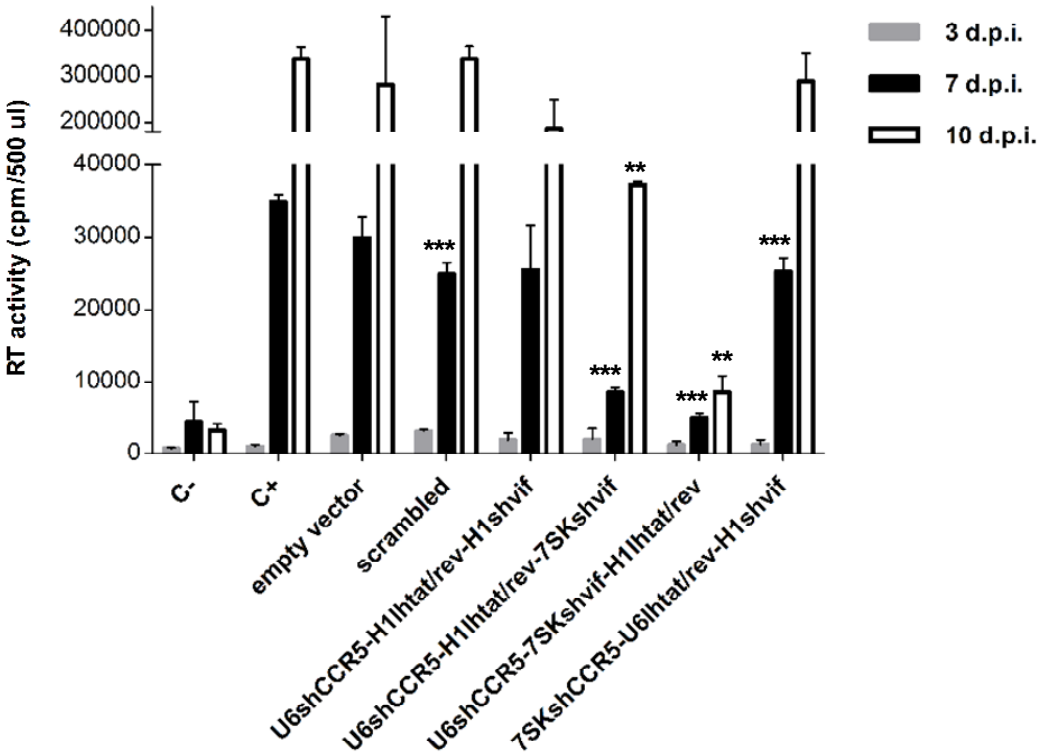


Table 5

Vector	EGFP+ cells (%)
empty vector	92 ± 9
scrambled	97 ± 5
U6e-shRNA	76 ± 12
7SKe-shRNA	75 ± 11
H1e-shRNA	76 ± 19

B

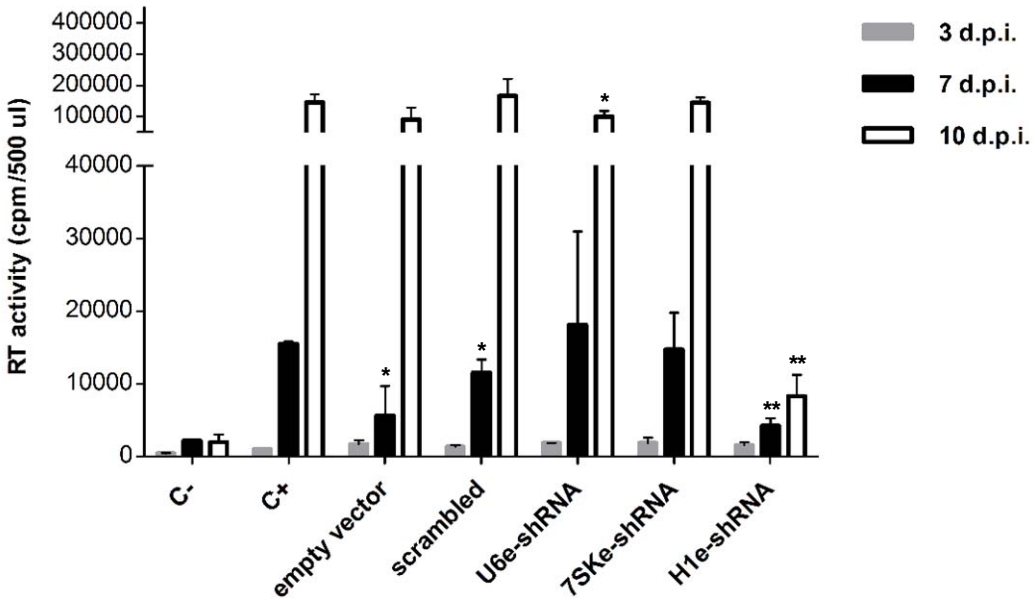


Figure 7.11. Inhibition of CXCR4-tropic HIV-1 infection in Jurkat T cells transduced with the combinatorial vectors. Jurkat cells were transduced with the pLL3.7 empty vector, the U6scrambled vector (scrambled) and either the triple cassette vectors (A) or the e-shRNA vectors (B). The percentage of EGFP+ cells at 3 days post-transduction is reported in Tables 4 and 5, respectively. Untransduced (C+) and transduced cells were infected with the HXBc2 Vpr+ Vpu+ Nef+ strain of HIV-1 and the culture supernatants, harvested at various time points [3, 7 or 10 days post infection (d.p.i.)], were tested for RT activity, as indicated. Error bars denote the standard deviation from 3 independent experiments (C-: untransduced and uninfected cells) (*, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; t-test, relative to C+ at the corresponding d.p.i.).

As shown in Figure 7.11A, HIV-1 replication was efficiently inhibited up to 7 days post-infection in cells transduced with two out of the four triple shRNA vectors (i.e. U6shCCR5-H1lhtat/rev-7SKshvif and U6shCCR5-7SKshvif-H1lhtat/rev), as compared either to untransduced infected cells (C+) or to scrambled- and empty vector-transduced cells. Furthermore, in the case of the U6shCCR5-7SKshvif-H1lhtat/rev vector, viral replication was nearly undetectable for as long as 10 days after infection, while, at the same time, the U6shCCR5-H1lhtat/rev-7SKshvif vector displayed a 10-fold decrease in viral replication. Strikingly, the U6shCCR5-H1lhtat/rev-H1shvif vector and the 7SKshCCR5-U6lhtat/rev-H1shvif vector behaved similar to the scrambled control. These data are in sharp contrast with results obtained with the luciferase knockdown assay (7.6). One explanation to this finding could be that the U6shCCR5-H1lhtat/rev-H1shvif and the 7SKshCCR5-U6lhtat/rev-H1shvif vectors undergo genetic instability during the transduction process. Supporting this hypothesis, we noticed that their titer, expressed as TU/ml, was reduced of more than 4-fold as compared to the one of the effective vectors (Figure 7.12B), while the RT activity in the supernatant of producer cells displayed only slight differences (Figure 7.12A). Furthermore, when transducing target cells at the same m.o.i., transduction efficiency of the U6shCCR5-H1lhtat/rev-H1shvif and the 7SKshCCR5-U6lhtat/rev-H1shvif vectors was markedly lower than that of the U6shCCR5-H1lhtat/rev-7SKshvif and U6shCCR5-7SKshvif-H1lhtat/rev vectors (Figure 7.12C).

On the other hand, results in Figure 7.11B demostrated that the H1-driven e-shRNA provided a robust resistance to HIV-1 replication for as long as 10 days post-infection. By contrast, neither the U6-driven nor the 7SK-driven e-shRNA displayed antiviral activity. These data are consistent with the results obtained by means of the luciferase assay (7.6).

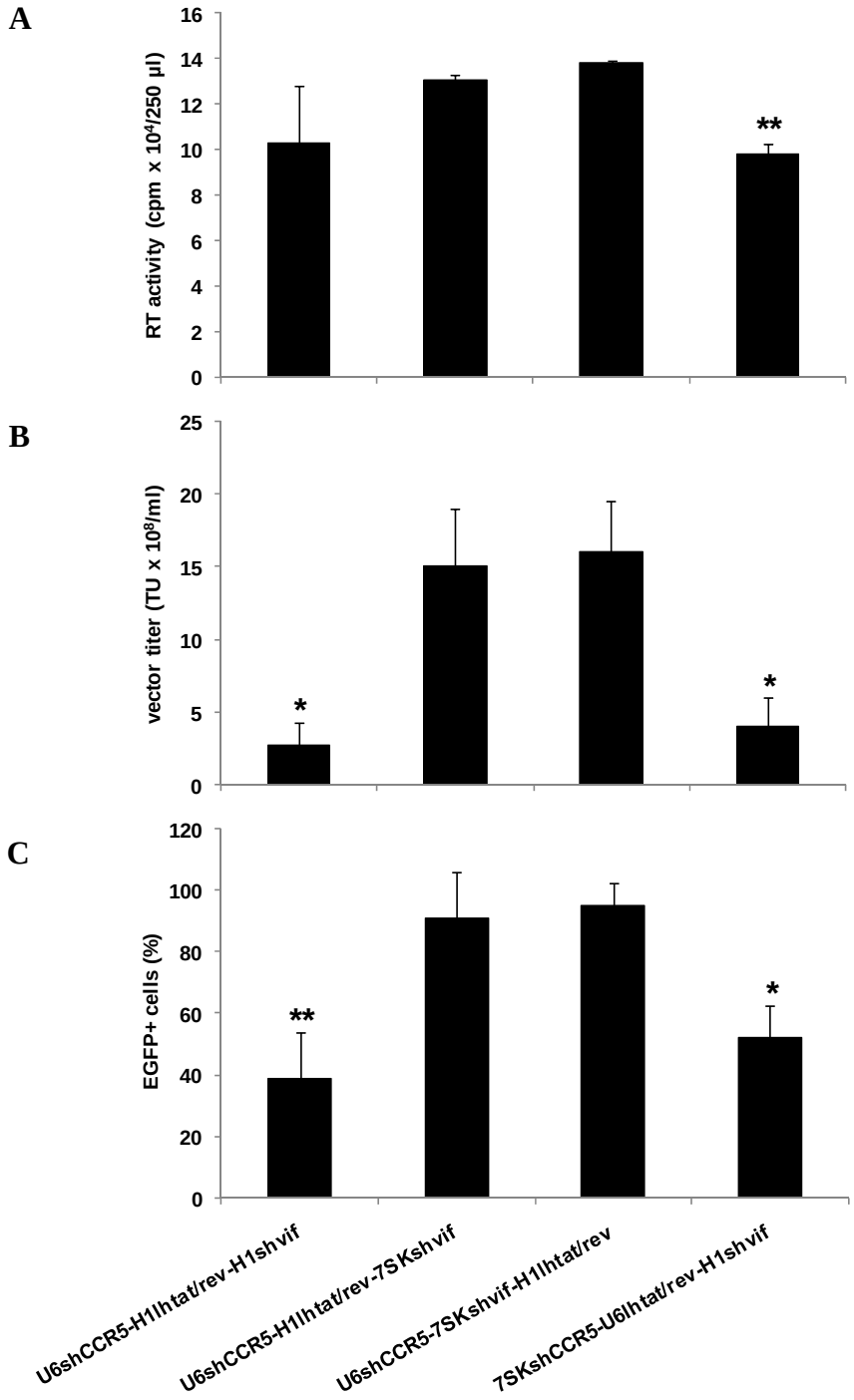


Figure 7.12. Differences in vector titer and transduction efficiency among the triple cassette vectors. (A) Equivalent amounts of supernatant from 293T cells, co-transfected with the packaging components along with each specified vector, were assayed for RT activity 2 days after transfection. (B) 293T cells were transduced with serial dilutions of the concentrated lentiviral stocks and, 72 h later, EGFP expression was assessed by flow cytometry, to determine vector titers. (C) Equivalent amounts of the indicated vectors were incubated with Jurkat T cells and, 72 h later, EGFP expression was analyzed by FACS, to determine transduction efficiency. The mean of three independent experiment is reported. The error bars represent the standard deviation (*, p-value < 0.05; **, p-value < 0.01; t-test, relative to U6shCCR5-7SKshvif-H1lhtat/rev).

7.9 Inhibition of HIV-1 replication in human primary CD4+ T lymphocytes by means of the combinatorial vectors

To confirm the results in a more physiologically relevant setting, we tested the ability of the combinatorial vectors to inhibit HIV-1 replication in primary CD4+ T cells, the major targets of HIV-1 infection *in vivo*. As a first step, CD4+ T lymphocytes were purified from the whole blood of healthy donors. To select the most appropriate purification protocol, T cell purity and yield obtained by using two different methods were compared. The first method involved the use of an antibody cocktail to separate unwanted cells from the CD4+ T cells, while the second one included a magnetic beads-based purification of CD4+ T cells. Details of the two protocols are described in the Materials and Methods section (6.2). The purity of the CD4+ T cell preparations and the presence of contamination with undesired cells (i.e. CD14+ monocytes, CD8+ T cells, CD19+ B cells) were assessed by flow cytometry, after staining with the appropriate antibodies. Given the results reported in Figure 7.13, the antibody cocktail-based technique was adopted thereafter to purify CD4+ T cells.

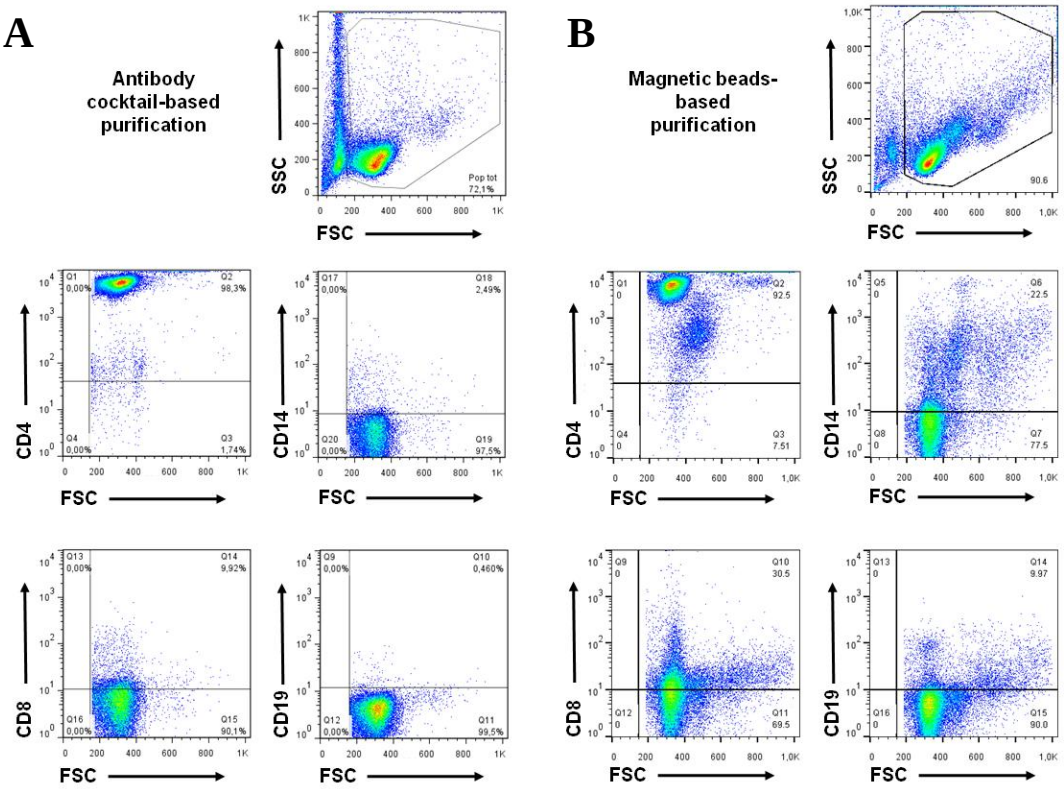


Figure 7.13. Purity of human primary CD4+ T lymphocytes prepared by different methods. CD4+ T cells were isolated by negative selection from buffy coats of healthy blood donors by using either an antibody cocktail (A) or magnetic beads (B) to separate unwanted cells from the CD4+ T cells. Purified cells were stained with antibodies to CD4, CD14 (monocyte/macrophage marker), CD8 (cytotoxic T lymphocyte marker) and CD19 (B lymphocyte marker). The expression of these surface markers was analyzed by flow cytometry. The results are exhibited as surface marker vs FSC dotplots with cell population in the live lymphocyte gate. The quadrant lines were defined by isotype control staining.

After purification, T cells were PHA-activated for 48 h, to allow lentiviral transduction. We verified that either prolonged exposure to PHA or PMA stimulation in addition to PHA didn't influence the activation status of T cells (data not shown).

Next, activated T cells were transduced with the combinatorial vectors that have been proven to be effective in *de novo* infected Jurkat cells (i.e. U6shCCR5-7SKshvif-H1lhtat/rev and H1e-shRNA) (7.8). Lymphocytes transduced with the empty vector served as control cells to measure uninhibited viral spread. Cells were spin-inoculated at an m.o.i. of 50 TU/cell in the presence of polybrene, achieving a percentage of EGFP+ cells typically up to 40% 72 h post-transduction (data not shown). Of note, although an m.o.i. of 50 was used for transduction with

different vectors, actual transduction efficiency appeared to vary from donor to donor, with the empty vector always displaying the higher efficiency. Moreover, our results showed that the use of retronectin didn't have a major impact on transduction efficiency, while in the absence of spin-inoculation, only a poor transduction efficiency was achieved (less than 10% on average, data not shown).

Following transduction, T cells were cultured in the presence of human IL-2 and CCR5 cell surface expression was monitored at different time points by flow cytometry. As indicated in Figure 7.14, CCR5 expression on the surface of lymphocytes greatly varied depending on the donor. Unfortunately, we couldn't detect major differences in CCR5 expression between combinatorial vector-transduced cells and empty vector-transduced cells (data not shown).

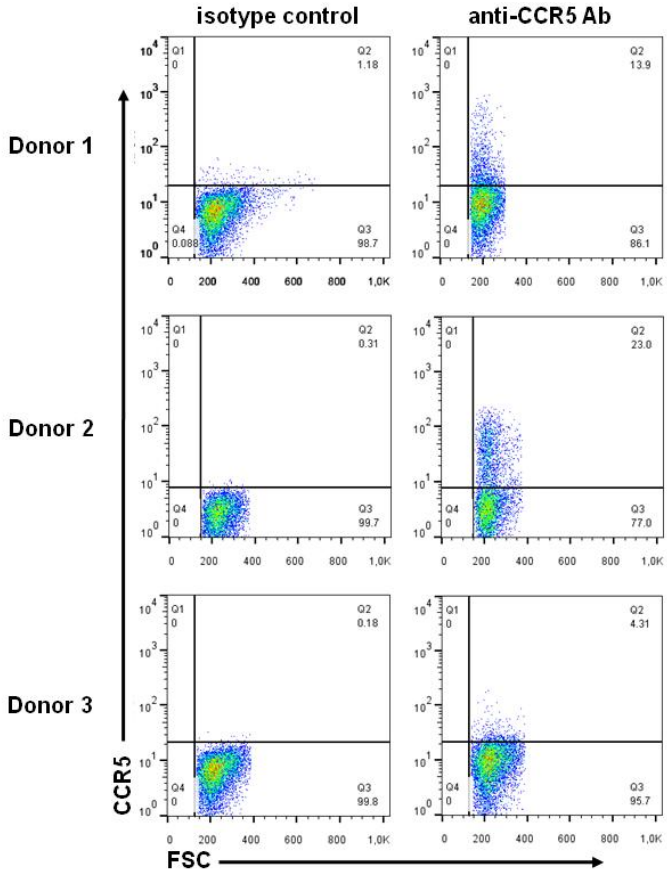
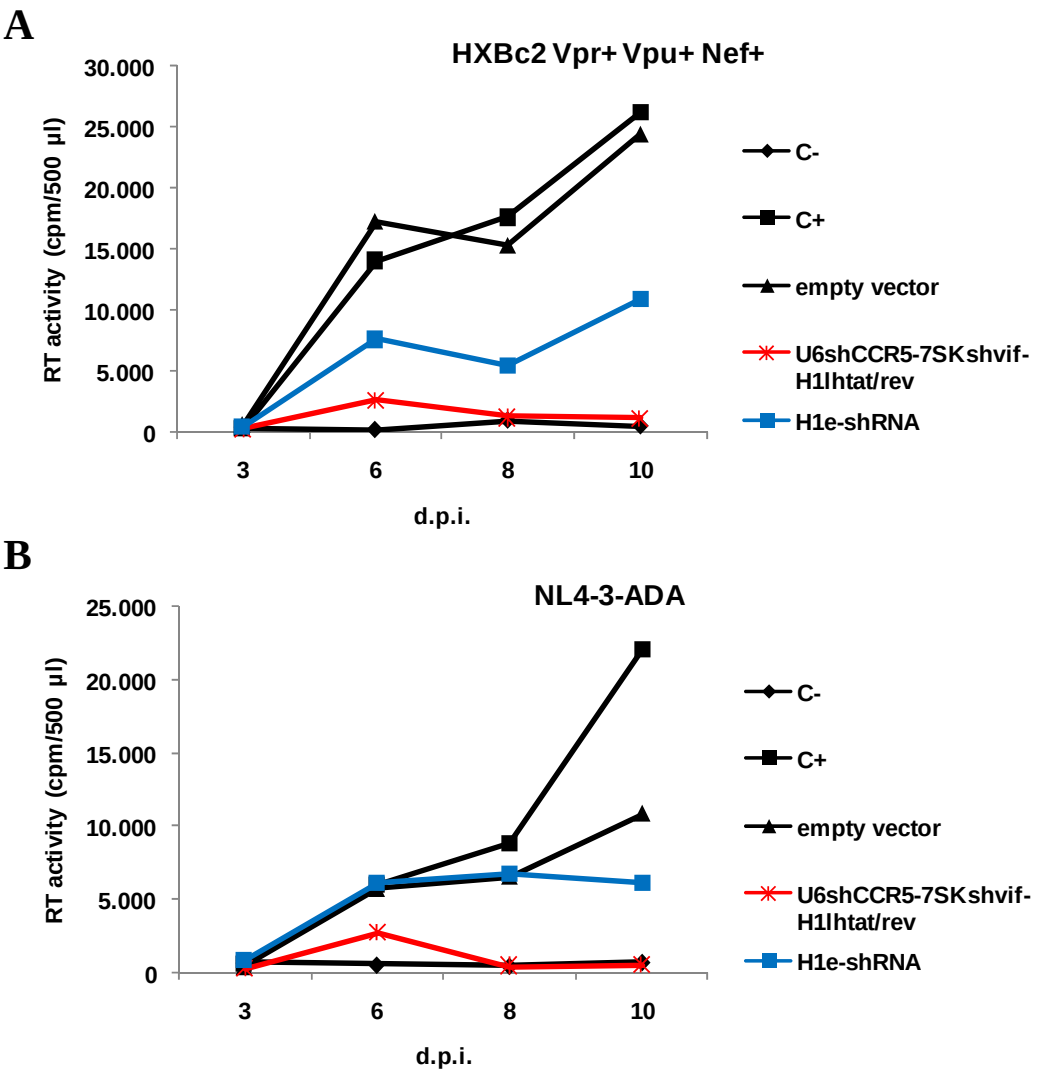


Figure 7.14. Interdonor variability of CCR5 surface expression on human primary CD4+ T lymphocytes. Purified CD4+ T lymphocytes were stimulated with PHA for 48 h and further cultured in the presence of IL-2 for 4 days before FACS analysis for CCR5 expression on the cell surface. The FACS results are presented as CCR5 vs FSC dotplots with cell populations in the live lymphocyte gate (typically > 70%). The quadrant lines were defined by isotype control staining, as indicated. Results from three donors (1, 2 and 3) are shown.

Taken together, these data prompted us to further investigate the antiviral activity of the combinatorial vectors in primary T cells upon HIV-1 challenge. To this end, CD4+ T lymphocytes were FACS sorted four days after transduction to obtain a pure EGFP+ population. The next day, cells were challenged with either the same X4-tropic HIV-1 strain previously described (HXBc2 Vpr+ Vpu+ Nef+) (7.8), or an R5-tropic HIV-1 strain (NL4-3-ADA). The latter virus is a laboratory-adapted HIV-1 strain with intact accessory genes (*vif*, *vpr*, *vpu*, *nef*). Importantly, infection with the HIV-1 NL4-3-ADA strain allowed us to evaluate the contribution of the siCCR5 sequence to the antiviral activity, in addition to the effect of the siRNAs against the *vif* and the *tat*/*rev* transcripts. Infection was performed by using equivalent RT units (10000 cpm) of each virus and viral

inhibition was determined by measuring the RT activity in the cell culture supernatants at different time points. In parallel, to exclude vector-related cytotoxicity, CD4+ T cells underwent the MTT cell viability assay 4 days after transduction.



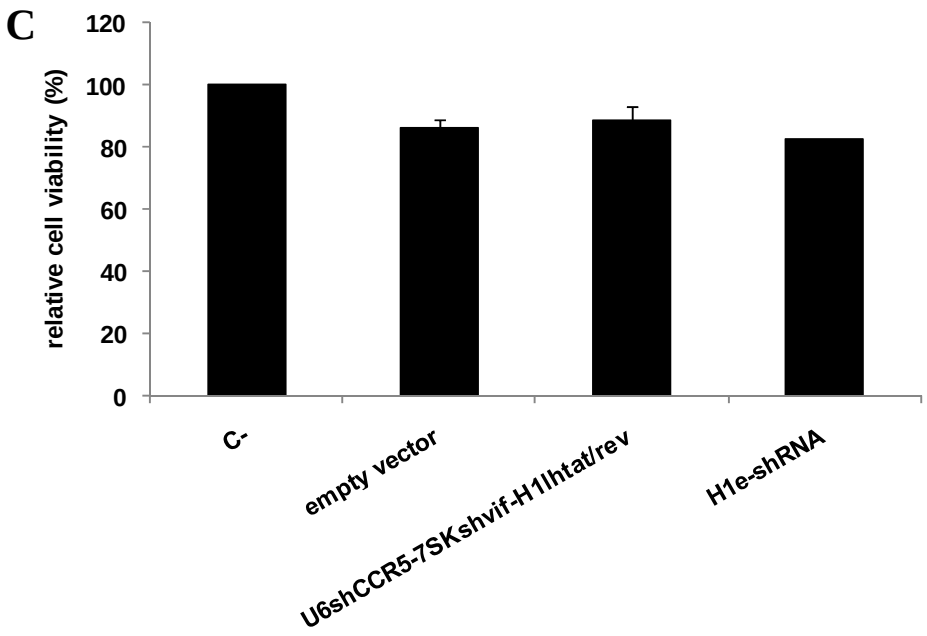


Figure 7.15. Inhibition of CXCR4- and CCR5-tropic HIV-1 infection in human primary CD4+ T lymphocytes transduced with the combinatorial vectors. PHA-stimulated purified CD4+ T lymphocytes were transduced with the pLL3.7 empty vector and the combinatorial vectors that displayed the highest antiviral activity upon Jurkat cell infection, as indicated. The transduced cells were cultured in IL-2-containing medium for 4 days before FACS sorting of EGFP+ cells. After additional 24 h, untransduced (C+) and FACS sorted EGFP+ T cells were challenged with either the HXBc2 Vpr+ Vpu+ Nef+ X4-tropic HIV-1 strain (A) or with the NL4-3-ADA R5-tropic HIV-1 strain (B). Culture supernatants were collected on the days indicated [3, 7 or 10 days post infection (d.p.i.)] and assayed for RT activity (C-: untransduced and uninfected cells). (C) Absence of combinatorial vector-related cytotoxicity in human primary CD4+ T lymphocytes. Cell viability of empty vector- and combinatorial vector-transduced T cells was measured by MTT assay 4 days after transduction. Cell viability was calculated by measuring the absorbance at 620 nm and normalizing it to untransduced cells. One representative experiment is shown (C-: untransduced and cells).

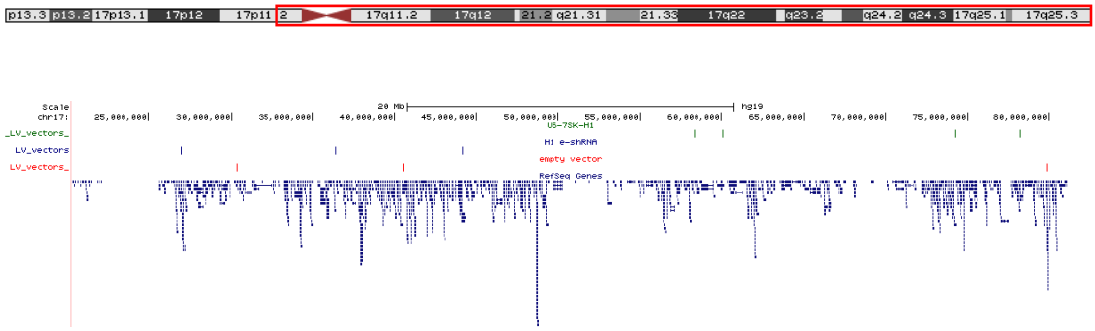
Both the triple shRNA vector and the e-shRNA vector provided a robust inhibition of viral replication when challenged with either the X4- or the R5-HIV-1 strain, relative to the controls [untransduced infected cells (C+) and empty vector-transduced cells] (Figure 7.15A-B). Further, viral inhibition was sustained for as long as 10 days after infection. Of note, the siRNAs expressed from the triple vector mediated the most potent antiviral effect in primary cells. Importantly, lymphocyte viability remained unaffected despite both the transduction process and the multiple exogenous siRNAs expression (Figure 7.15C).

7.10 Analysis of vector integration sites in human primary CD4+ T lymphocytes

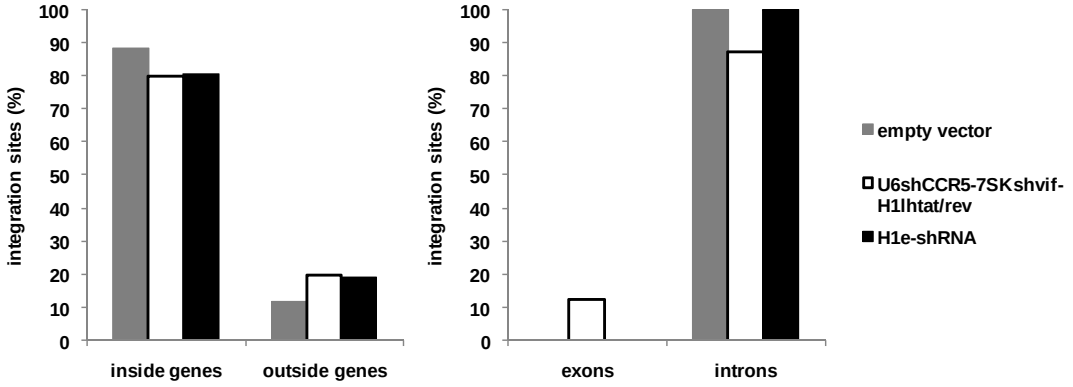
Considering that interactions between newly integrated vectors and the host genome could limit the reliability and safety of the gene therapy approach, we were interested in studying the integration profile of the combinatorial vectors in a physiologically relevant setting. To this end, human primary CD4+ T cells were transduced with the empty vector as well as with the combinatorial vectors displaying the highest anti-HIV-1 activity (i.e. U6shCCR5-7SKshvif-H1lhtat/rev and H1e-shRNA), as previously described (7.8). Transduction efficiency was up to 40%, depending on the vector and the experiment (data not shown). Genomic DNA was collected 5 to 10 days after transduction and vector-genome junctions were amplified by linker-mediated polymerase chain reaction (LM-PCR), inserted into a commercial plasmid and Sanger-sequenced. More than 200 raw reads were obtained up to date, which corresponded to 95 *bona* fide integration sites (ISs) univocally mapped onto the human genome. A total of 34, 30 and 31 ISs were identified in cells transduced with the empty vector, the triple cassette vector and the e-shRNA vector, respectively. A visual representation of an ISs cluster is shown in Figure 7.16A.

A

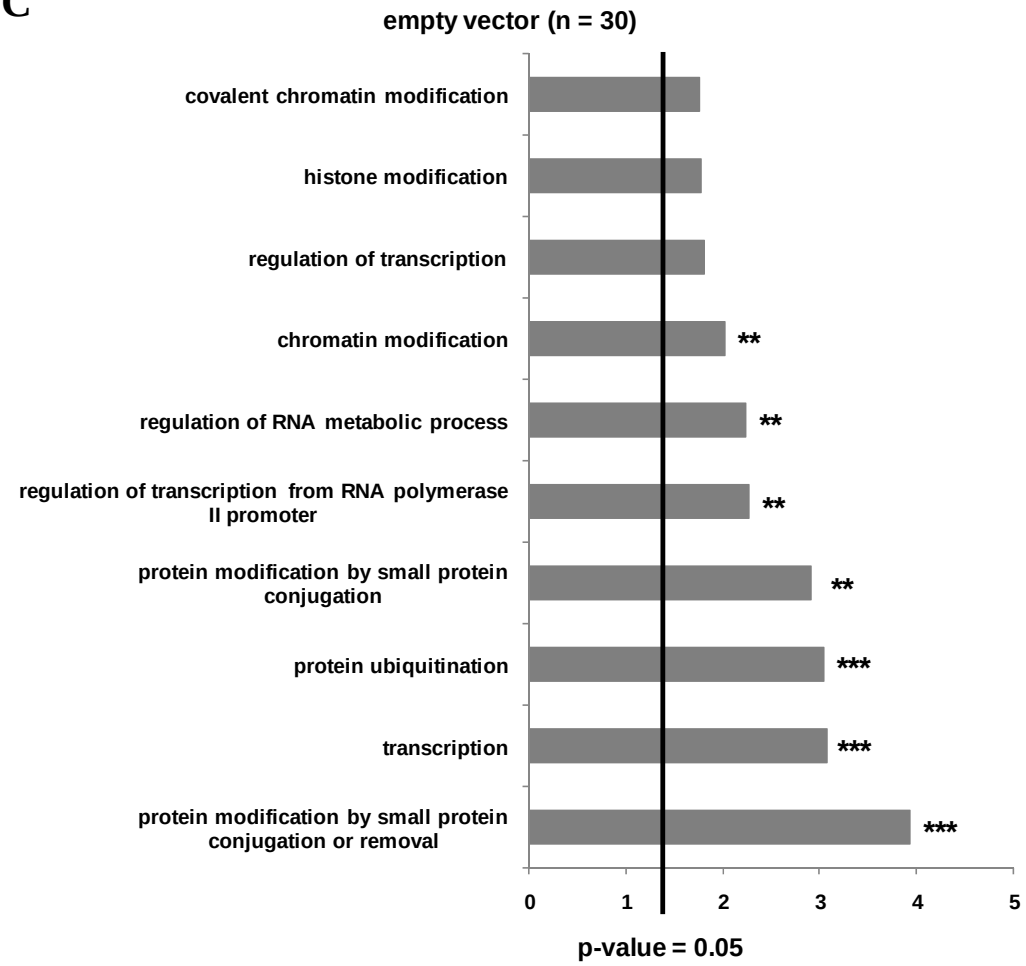
Chr17 (p11.2-q25.3)



B


integration sites (%)
100
90
80
70
60
50
40
30
20
10
0
inside genes
outside genes
exons
introns
empty vector
U6shCCR5-7SKshvif-H1lhtat/rev
H1e-shRNA

C


empty vector (n = 30)
covalent chromatin modification
histone modification
regulation of transcription
chromatin modification
**
regulation of RNA metabolic process
**
regulation of transcription from RNA polymerase II promoter
**
protein modification by small protein conjugation
**
protein ubiquitination
***
transcription
***
protein modification by small protein conjugation or removal
***
0
1
2
3
4
5
p-value = 0.05

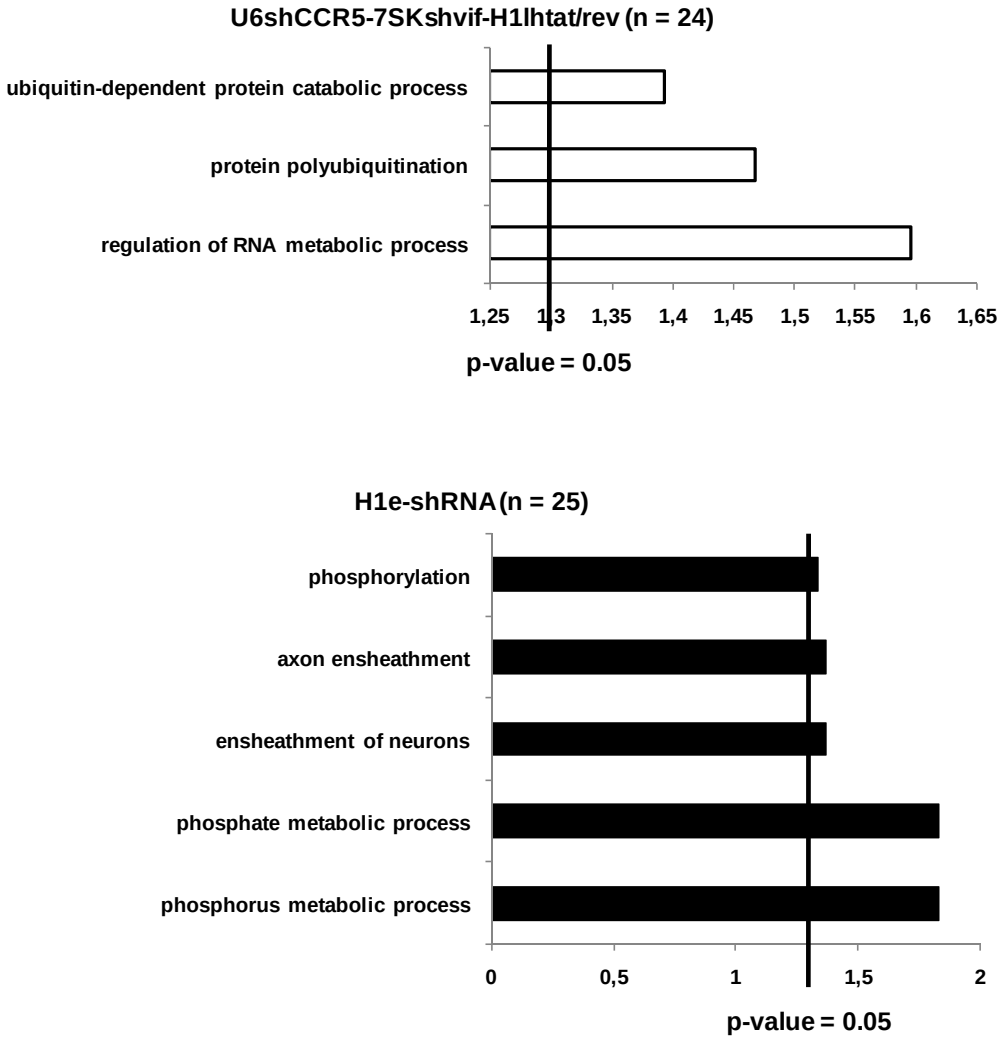


Figure 7.16. ISs distribution of the vectors in human primary CD4+ T lymphocytes. (A) Visual representation of an ISs cluster region on chromosome 17 (UCSC genome browser). Location of the cluster is indicated by a red rectangle on the chromosome (top). Zoom-in of the corresponding region (bottom). The base position feature (scale bar and nucleotide number) identifies the genomic coordinates of the displayed region. The RefSeq genes track shows known human protein-coding transcripts taken from the NCBI RNA reference sequences collection. (B) Distribution of ISs with respect to either RefSeq genes (left panel) or exons/introns (right panel). (C) Functional classification of genes targeted by ISs. The figure shows those function categories significantly over-represented among the target genes of vector integrations in CD4+ T cells (DAVID 6.7 software). A p-value < 0.05 was considered as statistically significant (1.3 in log scale, vertical line), (**, p-value < 0.01; ***, p-value < 0.001), (n) represents the total number of genes eligible for the analysis.

Despite the limited amount of annotated ISs, vectors showed a marked preference for intragenic insertion, and in particular for introns, since only 3 out of 79 intragenic ISs targeted exons (4%) (Figure 7.16B). Importantly, no major differences were detected between the empty vector and the combinatorial vectors, indicating that increased vector size, presence of multiple promoters or transgene sequences didn't alter the integration profile.

The Gene Ontology (GO) functional classification of the ISs target genes showed significant enrichment of specific categories. In particular, genes targeted by the empty vector integration are involved in protein modification, transcriptional regulation and RNA processing (p-value < 0.05) (Figure 7.16C, upper panel). The same or very similar functional categories (i.e. protein modification and regulation of RNA processing) were significantly enriched also in the set of genes hosting the triple vector integrations (p-value < 0.05) (Figure 7.16C, middle panel). Notably, the enrichment of related functional categories in the collection of genes targeted by the empty vector and the triple cassette vector was determined by different genes. Indeed, we never detected the same gene simultaneously hit by more than one vector (data not shown). On the contrary, the categories significantly over-represented in the e-shRNA vector dataset included phosphorus and phosphate metabolic processes, axon and neurons ensheathment and phosphorylation (p-value < 0.05) (Figure 7.16C, bottom panel).

8. DISCUSSION

Despite major advances in antiretroviral therapy, HIV-1 infection remains an epidemic cause of morbidity and mortality. Effective antiretroviral therapy often involves costly, multi-drug regimens that are not well tolerated by a significant percentage of patients, and even successful adherence to the therapy does not eradicate the virus, since a rapid rebound in HIV-1 levels can occur if therapy is discontinued.

An alternative, or complementary, approach to control HIV-1 replication is engineering the body's immune cells to be resistant to infection (Baltimore, 1988). In this context, over the past years, several anti-HIV-1 gene therapy approaches have been tested in hematopoietic stem cells (HSCs). These cells represent an attractive target, since they are the precursors of all the cells involved in HIV-1 pathogenesis (i.e. CD4+ T lymphocytes, macrophages, dendritic cells and microglia), and, thus, their genetic modification could protect the entire spectrum of susceptible cells. As HSCs proliferate extensively once they begin to contribute to blood cell production, they may function for years as an enduring source of HIV-1-resistant cells to replenish central and mucosal lymphoid organs. Thus, differently from lifelong drug therapy, a successful gene therapy protocol should be effective after a single treatment.

Recently, the development of HSC-based gene therapy approaches for HIV-1 infection received renewed attention thanks to the case of the Berlin patient. This HIV-1 infected individual was transplanted with HSCs from a donor homozygous for a deletion in the CCR5 gene (CCR5Δ32), as a treatment for leukemia. The CCR5Δ32 mutation prevents the CCR5 from appearing on the cell surface, without apparent adverse phenotypic effects. Since the majority of naturally occurring strains of HIV-1 uses CCR5 as a co-receptor for primary infection, individuals homozygous for the CCR5Δ32 are resistant to HIV-1 infection (Samson *et al.*, 1996). Notably, the Berlin patient is currently free from HIV-1 replication, while also having discontinued antiretroviral drug therapy (Hütter *et al.*, 2009; Hütter and Ganepola 2011a; Burke *et al.*, 2013). This proof-of-principle study demonstrated that HIV-1-resistant stem cells are capable of repopulating the immune system, providing a functional cure for HIV-1 infected patients.

The safety of numerous anti-HIV-1 genes has been demonstrated in previous HIV-1 stem cell gene therapy clinical trials, including both RNA- and protein-based inhibitors. To date, preclinical studies indicated that RNAi is the most potent RNA-based inhibitory mechanism available for therapeutic application, especially when induced via stable shRNA expression (Rossi *et al.*, 2007; Liu *et al.*, 2009). However, given the high rates of HIV-1 production in infected individuals and the ability of HIV-1 to generate escape variants, effective therapeutic application of RNAi for HIV-1 disease should involve the combination of multiple "genetic immunization" reagents directed against the virus (An *et al.*, 2007). Therefore, similar to combination approaches with antiviral drugs, multiple anti-HIV-1 genes inserted into a single gene therapy vector may offer stronger protection from viral infection and will have a greater chance of preventing resistance (Li *et al.*, 2005; Sano *et al.*, 2008; ter Brake *et al.*, 2008; Saayman *et al.*, 2008; Liu *et al.*, 2009; Walker *et al.*, 2012; Centlivre *et al.*, 2013). Combinatorial RNAi (co-RNAi) has been achieved by means of a number of different strategies, among which multiple promoter-shRNA cassettes and extended shRNAs (e-shRNAs) are the most extensively exploited. Although all of these approaches can result in efficient gene suppression, only few studies directly compare the efficacy of different co-RNAi platforms designed against the same targets, as well as the impact of promoter choice on the expression of a given RNAi effector.

In the present work, we developed lentiviral vectors expressing multiple anti-HIV-1 siRNAs, both as independent transcriptional units, and as an e-shRNA under the control of a single promoter. Our aims were to evaluate the impact of different promoters on siRNA silencing activity, to compare the antiviral efficacy of multiple co-RNAi platforms and to optimize vector design. The final goal of the research project is to use the most effective and safe vector(s) for transducing HSCs harvested from AIDS-related lymphoma (ARL) patients, that offer a unique opportunity to evaluate gene therapy strategies in an ethically acceptable clinical setting, as they often undergo autologous HSCs transplantation.

To achieve reliable HIV-1 inhibition, we sought to block multiple stages of the viral life cycle by targeting a combination of cellular and viral genes, namely the CCR5, *tat*, *rev* and *vif* genes. After having searched for active RNAi triggers in

literature, we selected shRNAs, generating one single siRNA, against either the CCR5 (shCCR5) (Liang *et al.*, 2010) or the *vif* transcript (shvif) (Lee *et al.*, 2005), and a long hairpin RNA (lhRNA), simultaneously giving rise to two different siRNAs against the *tat* and *rev* overlapping first exons (lhtat/rev) (Sano *et al.*, 2008). All these molecules proved to be highly effective against HIV-1 replication, since they target essential functions in the viral life cycle, including entry into target cells (shCCR5) (Liang *et al.*, 2010), gene expression (lhtat/rev) (Sano *et al.*, 2008) and infectivity of the newly produced particles (shvif) (Lee *et al.*, 2005). Moreover, the selected hairpins provide the following advantages: firstly, targeting of cellular co-factors such as CCR5 represents a valuable anti-escape approach, considering that the chance of resistance mutations in host transcripts is negligible compared to HIV-1 sequences; secondly, both the shvif and the lhtat/rev target conserved viral sequences, to prevent the selection of HIV-1 escape variants (Lee *et al.*, 2005; Sano *et al.*, 2008). In particular, the lhtat/rev, which produces two distinct siRNAs, is superior over a single shRNA in terms of longevity of viral inhibition.

In the first part of the work, we addressed the influence of the promoter used to express each RNAi trigger on its silencing activity. To this end, vectors expressing the shCCR5, the shvif or the lhtat/rev as a single transcriptional unit under the control of different human polymerase III promoters were constructed. Selected promoters included U6 and H1, which are the most widely exploited shRNA promoters, as well as the less frequently used 7SK promoter. These promoters are compact, active in many tissues and possess well known transcription start sites and termination signals. When assayed in the context of the luciferase knockdown assay, in which the RNAi target sequence is fused with the luciferase reporter gene, the U6 and the H1 promoters mediated an overall suppressive activity higher than that observed in the case of 7SK, irrespective of the RNAi effector. Furthermore, in our experimental settings, while the H1 and the U6 promoters displayed comparable activity in downregulating the CCR5 target sequence, H1 was superior to U6 in the case of the *vif* and *tat*/*rev* sequences. Literature data are controversial and only few studies directly compare the activity of different Pol III promoters. Results obtained by Mäkinen *et al.* indicated that the U6 promoter is more efficient than H1 in GFP silencing *in vitro*

and *in vivo*, leading to stable GFP knockdown in mouse brain for at least 9 months (Mäkinen *et al.*, 2006). By contrast, ter Brake and colleagues observed similar reporter gene knockdown when comparing the activity of the U6, the 7SK and the H1 promoters, driving the expression of shRNAs against the HIV-1 *pol*, *gag* and *tat*/*rev* transcripts (ter Brake *et al.*, 2008). Overall, our results suggested that the U6 and the H1 promoters are stronger than 7SK, but their activity may be influenced by the sequence, the structure and/or the length of the downstream RNAi trigger.

Consistent with these data, we demonstrated that CCR5 is downregulated by the shCCR5 both in the Cf2Th CD4+/CCR5+ canine thymocyte cell line and, more importantly, in human primary macrophages, with the U6 and the H1 promoters performing typically better than 7SK.

It is well known that CCR5 receptor density determines the susceptibility against HIV-1 transmission (Samson *et al.*, 1996; Michael *et al.*, 1997; Reynes *et al.*, 2001), and it has been shown that a linear decrease in CCR5 expression results in a logarithmic decrease in HIV-1 infection *in vitro* (Butticaz *et al.*, 2003). Indeed, CCR5Δ32 heterozygotes, whose CCR5 is reduced to 20-30% of wild-type levels, have a diminished susceptibility and progression of HIV-1 disease (Huang *et al.*, 1996; Meyer *et al.*, 1997; Quillent *et al.*, 1998). Given these considerations, the 3-fold CCR5 downregulation on primary macrophages we obtained in the presence of the shCCR5, as compared to untransduced cells, could have a marked clinical effect. Our results are consistent with those obtained by Liang and colleagues, that achieved an approximately 3-fold reduction of CCR5 expression in macrophages derived from human fetal liver (FL) CD34+ cells transduced with the shCCR5 molecule (Liang *et al.*, 2010). In that context, the observed CCR5 downregulation was sufficient to strongly inhibit viral replication after challenge with an R5-tropic HIV-1 strain (Liang *et al.*, 2010).

Previous studies performed by David Baltimore and collaborators identified an alternative shRNA against the CCR5, determining a 10-fold reduction in CCR5 expression on CD8+-depleted peripheral blood lymphocytes (Qin *et al.*, 2003). However, the expression of this shRNA from the U6 promoter resulted in cytotoxicity in primary cells, while the expression from other promoters highly

impaired shRNA potency, thus this antiviral molecule was no longer employed (An *et al.*, 2006, 2007).

Next, we tried to dissect the biological activity of the lhtat/rev by examining the effect of targeting either the HIV-1 Tat or Rev protein. Our results indicated that the lhtat/rev-mediated downregulation of either one of these proteins caused only a rather modest inhibition of viral gene expression in transfected or infected cells. On the one hand, a more pronounced inhibitory effect might have been accomplished by sorting of the lhRNA-transduced cells, as recently reported (Kalomoiris *et al.*, 2012). On the other hand, in our experimental conditions, only a tiny amount of Rev was required to express the *gag* gene, making it challenging to observe the effect of Rev dowregulation.

Compared with the shCCR5 and the lhtat/rev, the shvif displayed an overall less potent suppressive activity against its target in the luciferase assay. However, this siRNA proved to be broadly potent in previous studies, conferring protection against HIV-1 strains from multiple clades, by virtue of the high degree of target sequence conservation (Lee *et al.*, 2005). In addition, it is noteworthy that even small alteration in the expression or function of critical genes can influence the efficacy of HIV-1 transmission, viral load and collapse of the immune system (Ioannidis *et al.*, 2001; Alexander *et al.*, 2002; Fellay *et al.*, 2007; Rangel *et al.*, 2009; Hütter *et al.*, 2013).

Starting from these results, we developed anti-HIV-1 combinatorial vectors simultaneously expressing the above described siRNAs, according to different strategies. In general, the insertion of independent transcriptional units within a single vector offered the most reliable and predictable gene knockdown (Lambeth *et al.*, 2010; Centlivre *et al.*, 2013). Thus, we designed different vectors containing three independent transcriptional units, encoding the shCCR5, the shvif and the lhtat/rev, positioned in different orders with respect to each other within the vector framework. In some context, the same promoter was employed to drive the expression of multiple RNAi triggers, while in other context distinct promoters were adopted (triple vectors, i.e. U6shCCR5-H1lhtat/rev-H1shvif; U6shCCR5-H1lhtat/rev-7SKshvif; U6shCCR5-7SKshvif-H1lhtat/rev; 7SKshCCR5-U6lhtat/rev-H1shvif).

Considering that the use of multiple highly active RNA Pol III promoters can potentially flood the cell with shRNAs and abrogate the natural microRNA biogenesis pathway (Yi *et al.*, 2005; Grimm *et al.*, 2006; Boudreau *et al.*, 2008), we further adopted an e-shRNA molecule, as an alternative combinatorial approach. The e-shRNA simultaneously expresses the three siRNAs, targeting the CCR5, the *vif* and the *tat*/*rev* transcripts, under the control of a single promoter (e-shRNA vectors, i.e. U6e-shRNA; 7SKe-shRNA; H1e-shRNA).

Initial luciferase assays performed with the triple vectors indicated that, upon transfection of target cells, all the developed constructs were effective, as the individual shRNA activity was comparable for single and multiple shRNA vectors. However, a strikingly different pattern emerged when transduced lymphoblastoid Jurkat T cells were infected with a CXCR4-tropic strain of HIV-1. Indeed, in this latter case, only the U6shCCR5-H1lhtat/rev-7SKshvif and the U6shCCR5-7SKshvif-H1lhtat/rev vectors efficiently controlled HIV-1 replication up to 10 days post-infection, and the U6shCCR5-7SKshvif-H1lhtat/rev vector almost completely abolished viral infection, causing a 35-fold reduction of the total viral load. On the contrary, neither the U6shCCR5-H1lhtat/rev-H1shvif or the 7SKshCCR5-U6lhtat/rev-H1shvif conferred protection against HIV-1. Interestingly, these latter vectors displayed lower titer and transduction efficiency than the U6shCCR5-H1lhtat/rev-7SKshvif and the U6shCCR5-7SKshvif-H1lhtat/rev vectors, as measured by EGFP expression. One possible explanation for these results might be the occurrence of recombination events, resulting in deletion of one or more therapeutic cassettes. Supporting this hypothesis, previous studies have shown that the presence of repeated promoters causes frequent recombination within the lentiviral vector genome during the transduction process (ter Brake *et al.*, 2008), with a correlation between the length of the repeated sequence and the frequency of deletions (An *et al.*, 2001). The presence of repeated promoters may have a detrimental effect for the U6shCCR5-H1lhtat/rev-H1shvif vector, since it harbours two copies of H1. However, this is not likely to be a general rule, considering that a lentiviral vector expressing multiple anti-HIV-1 genes under the control of repeated Pol III promoters not only conferred strong resistance to HIV-1 infection (Li *et al.*, 2005), but it is also currently tested in phase I clinical trial (DiGiusto *et al.*, 2010). Thus, other key factors might

contribute to determine vector stability upon transduction of target cells, potentially influencing the reverse transcriptase activity. Among these, the local structure assumed by the vector genome, influenced by either the specific combination or the relative position of the shRNA cassettes, might play an important role.

By contrast, in the case of the e-shRNA vectors, the results obtained with both the luciferase assay and the viral challenge of transduced Jurkat cells clearly indicated that, among those tested, H1 was the only effective promoter in driving the expression of multiple siRNAs. Probably, in this context, promoter sequence directly impacts upon efficient processing and production of mature siRNAs, possibly through changes in their thermodynamic properties. To our knowledge, this is the first study directly comparing the impact of different promoters on e-shRNA expression.

The H1-driven e-shRNA determined a 20-fold reduction of the total viral load 10 days post-HIV-1 infection in Jurkat cells. Despite such a robust inhibition of viral replication, these data indicated that the e-shRNA vector was slightly less efficient than the most potent triple vector (i.e. U6shCCR5-7SKshvif-H1lhtat/rev). Eventually, some viral breakthrough occurred for both the triple and the e-shRNA vectors over the 4-weeks time course of HIV-1 replication in Jurkat cells (data not shown). This observation could reflect several mechanisms, such as sub-optimal inhibitory RNAi regimen, variability of inhibition over time and generation of escape or pseudo-escape viruses, that appear when a high virus input is tested (Qin *et al.*, 2003; Knoepfel *et al.*, 2012). In addition, it is important to remember that, on the one hand, the challenge virus was a CXCR4-tropic strain of HIV-1, thus only siRNAs targeting *vif* and *tat*/*rev*, but not CCR5, contributed to the observed antiviral activity. On the other hand, the EGFP+ cells were not sorted upon transduction, thus they are bathed continuously in virus produced by the unprotected cells.

The antiviral activity of the most promising triple and e-shRNA vectors (i.e. U6shCCR5-7SKshvif-H1lhtat/rev and H1e-shRNA) was further confirmed and extended in human primary CD4+ T lymphocytes, which are the major targets of HIV-1 infection *in vivo*. Consistent with previous data, the triple vector showed on average a 2-fold higher HIV-1 inhibition than the e-shRNA vector over the 10-

days time course of infection. In this context, we couldn't observe vector-mediated CCR5 downregulation, a finding most likely due to the low level of its expression on the surface of T cells.

Given that HSC transplantation is for a lifetime, the challenge is to identify shRNAs with sufficient potency to downregulate the desired genes over sustained lengths of time, but without toxicity to the cells bearing them. It has been previously shown that RNAi can cause toxic effects and trigger the induction of interferon response, leading to the loss of the transduced cells (Bridge *et al.*, 2003; Sledz *et al.*, 2003). In this regard, we included G:U mismatches in the passenger strand of the lhRNA and the e-shRNA, as they have been shown to prevent the activation of IFN-inducible genes, evading recognition by dsRNA-binding proteins in the cytoplasm (Sano *et al.*, 2008). In addition, our results ruled out a decrease in cell viability due to multiple siRNA expression, both in cell lines and in human primary T cells.

Apart from interferon induction, vectors integration profile represents an important safety issue, with significant consequences on their potential genotoxicity. Indeed, in the seminal clinical trials of HSC gene therapy for severe combined immunodeficiency (SCID) carried out by Cavazzana and colleagues, the clinical benefit of gene transfer was tempered by the occurrence of vector-related leukemia in some patients. In all these patients the therapeutic gammaretroviral vector integrated near proto-oncogenes and altered their expression, thereby promoting clonal T cell proliferation (Hacein-Bey-Abina *et al.*, 2003a,b, 2008). Several studies have probed the integration target site selection by gammaretroviruses, indicating that they preferentially integrate near transcriptional start sites and other transcriptional regulatory regions, such as CpG islands and DNAse I hypersensitive sites. This increases the risk to alter expression of nearby cellular genes, including proto-oncogenes and genes with regulatory functions (Wu *et al.*, 2003; Bushman *et al.*, 2005; Felice *et al.*, 2009 Montini *et al.*, 2009; Deichmann *et al.*, 2011; Sharma *et al.*, 2013). By contrast, lentiviral vectors integrate throughout the body of active genes, without showing a bias for promoter-proximal regions (De Palma *et al.*, 2005; Montini *et al.*, 2006; Zhou et al., 2010; Cattoglio C *et al.*, 2010; Cartier *et al.*, 2012). These data suggested that lentiviral vectors might have a reduced risk to alter transcriptional

regulation, even if they could still impact on the cellular transcriptome by inducing the formation of aberrantly spliced mRNAs (Cesana *et al.*, 2012; Moiani *et al.*, 2012). Consistent with literature data, our results, performed on a limited number of annotated ISs, indicated a vector integration preference for intragenic regions, especially for introns, in human primary T cells. Importantly, in this context, we didn't detect major differences between the vectors expressing multiple anti-HIV-1 siRNAs and the control vector. In addition, functional clustering analysis indicated that the triple and the control vectors share a common tendency to integrate within genes involved in protein modification and regulation of RNA metabolism. On the other hand, the e-shRNA vector preferentially targeted genes involved in phosphorous/phosphate metabolism and in neuron physiology. If supported by a deeper investigation, this latter evidence could have relevant implications for a possible use of the e-shRNA vector in gene therapy approaches for HIV-1 infection. Indeed, vector insertions within genes unrelated to both CD4+ T cell biology and transcriptional regulation might decrease the risk of adverse events. We are currently extending the collection of annotated ISs by next-generation sequencing of vector-genome junctions, in order to perform a detailed genome-wide analysis of vectors integration profile.

In conclusion, we described here new potent combinatorial approaches which may contribute to the development of an RNAi-based gene therapy strategy against HIV-1 infection. To our knowledge, this is the first time that multiple RNAi triggers targeting both viral and cellular genes are combined within a single vector. To date, anti HIV-1 gene therapy trials have mainly employed dominant negative proteins, ribozymes, antisense RNAs and decoys (reviewed in Hoxie and June, 2012). In 2008, the first shRNA against *tat*/*rev* was used in combination with a TAR decoy and a CCR5-ribozyme as an RNA-based HIV-1 gene therapy approach (DiGiusto *et al.*, 2010). This initial clinical result provided encouragement for the use of RNAi-based agents to fight HIV-1 infection and, currently, the first lentiviral vector expressing a triple combination of anti-HIV-1 shRNAs against the *pol* and *tat*/*rev* transcripts is approaching clinical testing (Knoepfel *et al.*, 2012).

In addition, our study highlighted some important strengths and pitfalls of different platforms used for multiple siRNAs delivery, providing valuable insights

for the design and application of reliable combinatorial RNAi to counteract HIV-1 replication.

Future studies will address vector efficacy and safety in HSCs, which represent the main targets of our anti-HIV gene therapy approach, as well as in humanized mouse models. As a result of this work, hopefully we should be able to build up a clinical protocol to genetically modify HSCs harvested from ARL patients. In this context, it is noteworthy to consider that, to fully exploit the therapeutic potential of the vectors, the gene therapy approach will be restricted to individuals with no detectable CXCR4-tropic virus.

9. REFERENCES

Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA. 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol 59(2):284–291.

Aiuti A, Biasco L, Scaramuzza S, Ferrua F, Cicalese MP, Baricordi C, Dionisio F, Calabria A, Giannelli S, Castiello MC, Bosticardo M, Evangelio C, Assanelli A, Casiraghi M, Di Nunzio S, Callegaro L, Benati C, Rizzardi P, Pellin D, Di Serio C, Schmidt M, Von Kalle C, Gardner J, Mehta N, Neduva V, Dow DJ, Galy A, Miniero R, Finocchi A, Metin A, Banerjee PP, Orange JS, Galimberti S, Valsecchi MG, Biffi A, Montini E, Villa A, Ciceri F, Roncarolo MG, Naldini L. 2013. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. Science 341(6148):1233151.

Alexander L, Aquino-DeJesus MJ, Chan M, Andiman WA. 2002. Inhibition of human immunodeficiency virus type 1 (HIV-1) replication by a two-amino-acid insertion in HIV-1 Vif from a nonprogressing mother and child. J Virol76(20):10533-9.

Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA. 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1.Science 272(5270):1955-8.

Allers K, Hütter G, Hofmann J, Loddenkemper C, Rieger K, Thiel E, Schneider T. 2011. Evidence for the cure of HIV infection by CCR5Δ32/Δ32 stem cell transplantation. Blood 117(10):2791-9.

An DS, Qin FX, Auyeung VC, Mao SH, Kung SK, Baltimore D, Chen IS. 2006. Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. Mol Ther 14(4):494-504.

An DS, Donahue RE, Kamata M, Poon B, Metzger M, Mao SH, Bonifacino A, Krouse AE, Darlix JL, Baltimore D, Qin FX, Chen IS. 2007. Stable reduction of CCR5 by RNAi through hematopoietic stem cell transplant in non-human primates. Proc Natl Acad Sci U S A 104(32):13110-5.

An W, Telesnitsky A. 2001. Frequency of direct repeat deletion in a human immunodeficiency virus type 1 vector during reverse transcription in human cells. Virology 286(2):475-82.

Anderson JS, Javien J, Nolta JA, Bauer G. 2009. Preintegration HIV-1 inhibition by a combination lentiviral vector containing a chimeric TRIM5 alpha protein, a CCR5 shRNA, and a TAR decoy. Mol Ther 17(12):2103-14.

Arts EJ, Hazuda DJ. 2012. HIV-1 antiretroviral drug therapy. Cold Spring HarbPerspect Med 2(4):a007161.

Baltimore D. 1988. Gene therapy. Intracellular immunization. Nature 335(6189):395-6.

Barré-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vézinet-Brun F, Rouzioux C, Rozenbaum W, Montagnier L. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220(4599):868-71.

Berkhout B. 2009. Toward a durable anti-HIV gene therapy based on RNA interference. Ann N Y Acad Sci 1175:3-14.

Biffi A, Montini E, Lorioli L, Cesani M, Fumagalli F, Plati T, Baldoli C, Martino S, Calabria A, Canale S, Benedicenti F, Vallanti G, Biasco L, Leo S, Kabbara N, Zanetti G, Rizzo WB, Mehta NA, Cicalese MP, Casiraghi M, Boelens JJ, Del Carro U, Dow DJ, Schmidt M, Assanelli A, Neduva V, Di Serio C, Stupka E, Gardner J, von Kalle C, Bordignon C, Ciceri F, Rovelli A, Roncarolo MG, Aiuti A, Sessa M, Naldini L. 2013. Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. Science 341(6148):1233158.

Bishop KN, Verma M, Kim EY, Wolinsky SM, Malim MH. 2008. APOBEC3G inhibits elongation of HIV-1 reverse transcripts. PLoS Pathog 4(12):e1000231.

Boudreau RL, Monteys AM, Davidson BL. 2008. Minimizing variables among hairpin-based RNAi vectors reveals the potency of shRNAs. RNA 14(9):1834-44.

Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R. 2003. Induction of an interferon response by RNAi vectors in mammalian cells. Nat Genet 34(3):263-4.

Brummelkamp TR, Bernards R, Agami R. 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science 296(5567):550-3.

Burke BP, Boyd MP, Impey H, Breton LR, Bartlett JS, Symonds GP, Hütter G. 2013. CCR5 as a Natural and Modulated Target for Inhibition of HIV. Viruses 6(1):54-68.

Bushman F, Lewinski M, Ciuffi A, Barr S, Leipzig J, Hannenhalli S, Hoffmann C. 2005. Genome-wide analysis of retroviral DNA integration. Nat Rev Microbiol 3(11):848-58.

Butticaz C, Ciuffi A, Muñoz M, Thomas J, Bridge A, Pebernard S, Iggo R, Meylan P, Telenti A. 2003. Protection from HIV-1 infection of primary CD4 T cells by CCR5 silencing is effective for the full spectrum of CCR5 expression. Antivir Ther 8(5):373-7.

Cartier N, Hacein-Bey-Abina S, Bartholomae CC, Bougnères P, Schmidt M, Kalle CV, Fischer A, Cavazzana-Calvo M, Aubourg P. 2012. Lentiviral hematopoietic cell gene therapy for X-linked adrenoleukodystrophy. Methods Enzymol 507:187-98.

Cattoglio C, Facchini G, Sartori D, Antonelli A, Miccio A, Cassani B, Schmidt M, von Kalle C, Howe S, Thrasher AJ, Aiuti A, Ferrari G, Recchia A, Mavilio F. 2007. Hot spots of retroviral integration in human CD34+ hematopoietic cells. Blood 110(6):1770-8.

Cattoglio C, Pellin D, Rizzi E, Maruggi G, Corti G, Miselli F, Sartori D, Guffanti A, Di Serio C, Ambrosi A, De Bellis G, Mavilio F. 2010. High-definition mapping of retroviral integration sites identifies active regulatory elements in human multipotent hematopoietic progenitors. Blood 116(25):5507-17.

Cattoglio C, Maruggi G, Bartholomae C, Malani N, Pellin D, Cocchiarella F, Magnani Z, Ciceri F, Ambrosi A, von Kalle C, Bushman FD, Bonini C, Schmidt M, Mavilio F, Recchia A. 2010. High-definition mapping of retroviral integration sites defines the fate of allogeneic T cells after donor lymphocyte infusion. PLoS One 5(12):e15688.

Centlivre M, Legrand N, Klamer S, Liu YP, Jasmijn von Eije K, Bohne M, Rijnstra ES, Weijer K, Blom B, Voermans C, Spits H, Berkhout B. 2013. Preclinical in vivo evaluation of the safety of a multi-shRNA-based gene therapy against HIV-1. Mol Ther Nucleic Acids 2:e120.

Cesana D, Sgualdino J, Rudilosso L, Merella S, Naldini L, Montini E. 2012. Whole transcriptome characterization of aberrant splicing events induced by lentiviral vector integrations. J Clin Invest 122(5):1667-76.

Chan E, Schaller T, Eddaoudi A, Zhan H, Tan CP, Jacobsen M, Thrasher AJ, Towers GJ, Qasim W. 2012. Lentiviral gene therapy against human immunodeficiency virus type 1, using a novel human TRIM21-cyclophilin A restriction factor. Hum Gene Ther 23(11):1176-85.

Chertova E, Chertov O, Coren LV, Roser JD, Trubey CM, Bess JW Jr, Sowder RC 2nd, Barsov E, Hood BL, Fisher RJ, Nagashima K, Conrads TP, Veenstra TD, Lifson JD, Ott DE. 2006. Proteomic and biochemical analysis of purified human immunodeficiency virus type 1 produced from infected monocyte-derived macrophages. J Virol 80(18):9039-52.

Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C, Sodroski J. 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 85(7):1135-48.

Cohen J. 2011. HIV/AIDS research. Tissue says blood is misleading, confusing HIV cure efforts. Science 334(6063):1614.

Davidson BL, McCray PB Jr. 2011. Current prospects for RNA interference-based therapies. Nat Rev Genet 12(5):329-40.

De Palma M, Montini E, Santoni de Sio FR, Benedicenti F, Gentile A, Medico E, Naldini L. 2005. Promoter trapping reveals significant differences in integration site selection between MLV and HIV vectors in primary hematopoietic cells. Blood105(6):2307-15.

Deichmann A, Brugman MH, Bartholomae CC, Schwarzwaelder K, Verstegen MM, Howe SJ, Arens A, Ott MG, Hoelzer D, Seger R, Grez M, Hacein-Bey-Abina S, Cavazzana-Calvo M, Fischer A, Paruzynski A, Gabriel R, Glimm H, Abel U, Cattoglio C, Mavilio F, Cassani B, Aiuti A, Dunbar CE, Baum C, Gaspar HB, Thrasher AJ, von Kalle C, Schmidt M, Wagemaker G. 2011. Insertion sites in engrafted cells cluster within a limited repertoire of genomic areas after gammaretroviral vector gene therapy. Mol Ther19(11):2031-9.

Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR. 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature 381(6584):661-6.

DiGiusto DL, Krishnan A, Li L, Li H, Li S, Rao A, Mi S, Yam P, Stinson S, Kalos M, Alvarnas J, Lacey SF, Yee JK, Li M, Couture L, Hsu D, Forman SJ, Rossi JJ, Zaia JA. 2010. RNA-based gene therapy for HIV with lentiviral vector-modified CD34(+) cells in patients undergoing transplantation for AIDS-related lymphoma. SciTransl Med 2(36):36ra43.

Di Nunzio F, Félix T, Arhel NJ, Nisole S, Charneau P, Beignon AS. 2012. HIV-derived vectors for therapy and vaccination against HIV. Vaccine 30(15):2499-509.

Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. 1998. A third-generation lentivirus vector with a conditional packaging system. J Virol 72(11):8463-71.

Eekels JJ, Geerts D, Jeeninga RE, Berkhout B. 2011. Long-term inhibition of HIV-1 replication with RNA interference against cellular co-factors. Antiviral Res 89(1):43-53.

Egelhofer M, Brandenburg G, Martinius H, Schult-Dietrich P, Melikyan G, Kunert R, Baum C, Choi I, Alexandrov A, von Laer D. 2004. Inhibition of human immunodeficiency virus type 1 entry in cells expressing gp41-derived peptides. J Virol78(2):568-75.

Engelman A, Cherepanov P. 2008. The lentiviral integrase binding protein LEDGF/p75 and HIV-1 replication. PLoS Pathog4(3):e1000046.

Felice B, Cattoglio C, Cittaro D, Testa A, Miccio A, Ferrari G, Luzi L, Recchia A, Mavilio F. 2009. Transcription factor binding sites are genetic determinants of retroviral integration in the human genome. PLoS One4(2):e4571.

Fellay J, Shianna KV, Ge D, Colombo S, Ledergerber B, Weale M, Zhang K, Gumbs C, Castagna A, Cossarizza A, Cozzi-Lepri A, De Luca A, Easterbrook P, Francioli P, Mallal S, Martinez-Picado J, Miro JM, Obel N, Smith JP, Wyniger J, Descombes P, Antonarakis SE, Letvin NL, McMichael AJ, Haynes BF, Telenti A, Goldstein DB. 2007. A whole-genome association study of major determinants for host control of HIV-1. Science 317(5840):944-7.

Feng S, Holland EC. 1988. HIV-1 tat trans-activation requires the loop sequence within tar. Nature 334(6178):165-7.

Feng Y, Broder CC, Kennedy PE, Berger EA. 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 272(5263):872-7.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391(6669):806-11.

Ganser-Pornillos BK, Yeager M, Sundquist WI. 2008. The structural biology of HIV assembly. Curr Opin Struct Biol18(2):203-17.

Gaspar HB, Cooray S, Gilmour KC, Parsley KL, Adams S, Howe SJ, Al Ghonaium A, Bayford J, Brown L, Davies EG, Kinnon C, Thrasher AJ. 2011. Long-term persistence of a polyclonal T cell repertoire after gene therapy for X-linked severe combined immunodeficiency. SciTransl Med 3(97):97ra79.

GLOBAL REPORT UNAIDS report on the global AIDS epidemic 2013. http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2013/gr2013/UNAIDS_Global_Report_2013_en.pdf

Göttlinger HG, Dorfman T, Sodroski JG, Haseltine WA. 1991. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. Proc Natl Acad Sci U S A 88(8):3195-9.

Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA. 2006. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441(7092):537-41.

Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A. 2003a. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 348(3):255-6.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JI, de Saint Basile G, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M. 2003b. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302(5644):415-9.

Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, Clappier E, Caccavelli L, Delabesse E, Beldjord K, Asnafi V, MacIntyre E, Dal Cortivo L, Radford I, Brousse N, Sigaux F, Moshous D, Hauer J, Borkhardt A, Belohradsky BH, Wintergerst U, Velez MC, Leiva L, Sorensen R, Wulffraat N, Blanche S, Bushman FD, Fischer A, Cavazzana-Calvo M. 2008. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J Clin Invest118(9):3132-42.

Helseth E, Kowalski M, Gabuzda D, Olshevsky U, Haseltine W, Sodroski J. 1990. Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. J Virol 64(5):2416-20.

Ho DD, Bieniasz PD. 2008. HIV-1 at 25. Cell 133(4):561-5.

Holt N, Wang J, Kim K, Friedman G, Wang X, Taupin V, Crooks GM, Kohn DB, Gregory PD, Holmes MC, Cannon PM. 2010. Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. Nat Biotechnol 28(8):839-47.

Hoxie JA, June CH. 2012. Novel cell and gene therapies for HIV. Cold Spring HarbPerspect Med 2(10).

Huang Y, Paxton WA, Wolinsky SM, Neumann AU, Zhang L, He T, Kang S, Ceradini D, Jin Z, Yazdanbakhsh K, Kunstman K, Erickson D, Dragon E, Landau NR, Phair J, Ho DD, Koup RA. 1996. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. Nat Med 2(11):1240-3.

Hütter G, Nowak D, Mossner M, Ganepola S, Müssig A, Allers K, Schneider T, Hofmann J, Kücherer C, Blau O, Blau IW, Hofmann WK, Thiel E. 2009. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. N Engl J Med 360(7):692-8.

Hütter G, Ganepola S. 2011a. Eradication of HIV by transplantation of CCR5-deficient hematopoietic stem cells. ScientificWorldJournal 11:1068-76.

Hütter G, Ganepola S. 2011b. The CCR5-delta32 polymorphism as a model to study host adaptation against infectious diseases and to develop new treatment strategies. ExpBiol Med (Maywood) 236(8):938-43.

Hütter G, Blüthgen C, Neumann M, Reinwald M, Nowak D, Klüter H. 2013. Coregulation of HIV-1 dependency factors in individuals heterozygous to the CCR5-delta32 deletion. AIDS Res Ther 10(1):26.

Ioannidis JP, Rosenberg PS, Goedert JJ, Ashton LJ, Benfield TL, Buchbinder SP, Coutinho RA, Eugen-Olsen J, Gallart T, Katzenstein TL, Kostrikis LG, Kuipers H, Louie LG, Mallal SA, Margolick JB, Martinez OP, Meyer L, Michael NL, Operskalski E, Pantaleo G, Rizzardi GP, Schuitemaker H, Sheppard HW, Stewart GJ, Theodorou ID, Ullum H, Vicenzi E, Vlahov D, Wilkinson D, Workman C, Zagury JF, O'Brien TR; International Meta-Analysis of HIV Host Genetics. 2001. Effects of CCR5-Delta32, CCR2-64I, and SDF-1 3'A alleles on HIV-1 disease

progression: An international meta-analysis of individual-patient data. Ann Intern Med135(9):782-95.

Jacobson JM. 2013. HIV gene therapy research advances. Blood 121(9):1483-4.

Jawetz E, Melnick JL, Adelberg EA. 1980. Serologic diagnosis and immunologic detection of virus infection. Review of Medical Microbiology. Lange Medical Publications, Los Altos, California, pp.371-285.

Kalomoiris S, Lawson J, Chen RX, Bauer G, Nolta JA, Anderson JS. 2012. CD25 preselective anti-HIV vectors for improved HIV gene therapy. Hum Gene Ther Methods 23(6):366-75.

Kitchen SG, Shimizu S, An DS. 2011. Stem cell-based anti-HIV gene therapy.Virology 411(2):260-72.

Knoepfel SA, Centlivre M, Liu YP, Boutimah F, Berkhout B. 2012. Selection of RNAi-based inhibitors for anti-HIV gene therapy. World J Virol 1(3):79-90.

LaBonte JA, Madani N, Sodroski J. 2003. Cytolysis by CCR5-using human immunodeficiency virus type 1 envelope glycoproteins is dependent on membrane fusion and can be inhibited by high levels of CD4 expression. J Virol 77(12):6645-59.

Lambeth LS, Van Hateren NJ, Wilson SA, Nair V. 2010. A direct comparison of strategies for combinatorial RNA interference. BMC Mol Biol 11:77.

Lee SK, Dykxhoorn DM, Kumar P, Ranjbar S, Song E, Maliszewski LE, François-Bongarçon V, Goldfeld A, Swamy NM, Lieberman J, Shankar P. 2005. Lentiviral delivery of short hairpin RNAs protects CD4 T cells from multiple clades and primary isolates of HIV. Blood 106(3):818-26.

Leonard JN, Schaffer DV. 2006. Antiviral RNAi therapy: emerging approaches for hitting a moving target. Gene Ther 13(6):532-40.

Levine BL, Humeau LM, Boyer J, MacGregor RR, Rebello T, Lu X, Binder GK, Slepushkin V, Lemiale F, Mascola JR, Bushman FD, Dropulic B, June CH. 2006. Gene transfer in humans using a conditionally replicating lentiviral vector. ProcNatlAcadSci U S A 103(46):17372-7.

Li L, Krymskaya L, Wang J, Henley J, Rao A, Cao LF, Tran CA, Torres-Coronado M, Gardner A, Gonzalez N, Kim K, Liu PQ, Hofer U, Lopez E, Gregory PD, Liu Q, Holmes MC, Cannon PM, Zaia JA, DiGiusto DL. 2013.

Genomic editing of the HIV-1 coreceptor CCR5 in adult hematopoietic stem and progenitor cells using zinc finger nucleases. MolTher 21(6):1259-69.

Li MJ, Bauer G, Michienzi A, Yee JK, Lee NS, Kim J, Li S, Castanotto D, Zaia J, Rossi JJ. 2003. Inhibition of HIV-1 infection by lentiviral vectors expressing Pol III-promoted anti-HIV RNAs. MolTher 8(2):196-206.

Li MJ, Kim J, Li S, Zaia J, Yee JK, Anderson J, Akkina R, Rossi JJ. 2005. Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. Mol Ther 12(5):900-9.

Liang M, Kamata M, Chen KN, Pariente N, An DS, Chen IS. 2010. Inhibition of HIV-1 infection by a unique short hairpin RNA to chemokine receptor 5 delivered into macrophages through hematopoietic progenitor cell transduction. J Gene Med 12(3):255-65.

Liu YP, Haasnoot J, Berkhout B. 2007. Design of extended short hairpin RNAs for HIV-1 inhibition. Nucleic Acids Res 35(17):5683-93.

Liu YP, von Eije KJ, Schopman NC, Westerink JT, ter Brake O, Haasnoot J, Berkhout B. 2009. Combinatorial RNAi against HIV-1 using extended short hairpin RNAs. MolTher 17(10):1712-23.

Liu YP, Berkhout B. 2011. miRNA cassettes in viral vectors: problems and solutions. BiochimBiophysActa 1809(11-12):732-45.

Maertens G, Cherepanov P, Pluymers W, Busschots K, De Clercq E, Debyser Z, Engelborghs Y. 2003. LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. J Biol Chem 278(35):33528-39.

Mäkinen PI, Koponen JK, Kärkkäinen AM, Malm TM, Pulkkinen KH, Koistinaho J, Turunen MP, Ylä-Herttuala S. 2006. Stable RNA interference: comparison of U6 and H1 promoters in endothelial cells and in mouse brain. J Gene Med 8(4):433-41.

Malim MH, Hauber J, Le SY, Maizel JV, Cullen BR. 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature 338(6212):254-7.

Manche L, Green SR, Schmedt C, Mathews MB. 1992. Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol Cell Biol 12(11):5238-48.

Mangeat B, Turelli P, Caron G, Friedli M, Perrin L, Trono D. 2003. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. Nature 424(6944):99-103.

Manjunath N, Wu H, Subramanya S, Shankar P. 2009. Lentiviral delivery of short hairpin RNAs. Adv Drug Deliv Rev 61(9):732-45.

Margolis AM, Heverling H, Pham PA, Stolbach A. 2013. A Review of the Toxicity of HIV Medications. J Med Toxicol [Epub ahead of print].

Martinson JJ, Chapman NH, Rees DC, Liu YT, Clegg JB. 1997. Global distribution of the CCR5 gene 32-basepair deletion. Nat Genet 16(1):100-3.

Mbisa JL, Barr R, Thomas JA, Vandegraaff N, Dorweiler IJ, Svarovskaia ES, Brown WL, Mansky LM, Gorelick RJ, Harris RS, Engelman A, Pathak VK. 2007. Human immunodeficiency virus type 1 cDNAs produced in the presence of APOBEC3G exhibit defects in plus-strand DNA transfer and integration. J Virol 81(13):7099-110.

McGarrity GJ, Hoyah G, Winemiller A, Andre K, Stein D, Blick G, Greenberg RN, Kinder C, Zolopa A, Binder-Scholl G, Tebas P, June CH, Humeau LM, Rebello T. 2013. Patient monitoring and follow-up in lentiviral clinical trials. J Gene Med 15(2):78-82.

McIntyre GJ, Yu YH, Tran A, Jaramillo AB, Arndt AJ, Millington ML, Boyd MP, Elliott FA, Shen SW, Murray JM, Applegate TL. 2009. Cassette deletion in multiple shRNAlentiviral vectors for HIV-1 and its impact on treatment success.Virol J 6:184.

McIntyre GJ, Arndt AJ, Gillespie KM, Mak WM, Fanning GC. 2011. A comparison of multiple shRNA expression methods for combinatorial RNAi. Genet Vaccines Ther 9(1):9.

Meyer L, Magierowska M, Hubert JB, Rouzioux C, Deveau C, Sanson F, Debre P, Delfraissy JF, Theodorou I. 1997. Early protective effect of CCR-5 delta 32 heterozygosity on HIV-1 disease progression: relationship with viral load. The SEROCO Study Group. AIDS 11(11):F73-8.

Michael NL, Louie LG, Rohrbaugh AL, Schultz KA, Dayhoff DE, Wang CE, Sheppard HW. 1997. The role of CCR5 and CCR2 polymorphisms in HIV-1 transmission and disease progression.HIV-1-resistance phenotype conferred by combination of two separate inherited mutations of CCR5 gene. Nat Med 3(10):1160-2.

Mitsuyasu RT, Merigan TC, Carr A, Zack JA, Winters MA, Workman C, Bloch M, Lalezari J, Becker S, Thornton L, Akil B, Khanlou H, Finlayson R, McFarlane R, Smith DE, Garsia R, Ma D, Law M, Murray JM, von Kalle C, Ely JA, Patino SM, Knop AE, Wong P, Todd AV, Haughton M, Fuery C, Macpherson JL, Symonds GP, Evans LA, Pond SM, Cooper DA. 2009. Phase 2 gene therapy trial of an anti-HIV ribozyme in autologous CD34+ cells. Nat Med 15(3):285-92.

Mitsuyasu RT, Zack JA, Macpherson JL, Symonds GP. 2011. Phase I/II Clinical Trials Using Gene-Modified Adult Hematopoietic Stem Cells for HIV: Lessons Learnt. Stem Cells Int 2011:393698.

Moiani A, Paleari Y, Sartori D, Mezzadra R, Miccio A, Cattoglio C, Cocchiarella F, Lidonnici MR, Ferrari G, Mavilio F. 2012. Lentiviral vector integration in the human genome induces alternative splicing and generates aberrant transcripts. J Clin Invest 122(5):1653-66.

Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C, Sergi Sergi L, Benedicenti F, Ambrosi A, Di Serio C, Doglioni C, von Kalle C, Naldini L. 2006. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. Nat Biotechnol 24(6):687-96.

Montini E, Cesana D, Schmidt M, Sanvito F, Bartholomae CC, Ranzani M, Benedicenti F, Sergi LS, Ambrosi A, Ponzoni M, Doglioni C, Di Serio C, von Kalle C, Naldini L. 2009. The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. J Clin Invest 119(4):964-75.

Naldini L. 2011. Ex vivo gene transfer and correction for cell-based therapies. Nat Rev Genet 12(5):301-15.

Neagu MR, Ziegler P, Pertel T, Strambio-De-Castillia C, Grütter C, Martinetti G, Mazzucchelli L, Grütter M, Manz MG, Luban J. 2009. Potent inhibition of HIV-1 by TRIM5-cyclophilin fusion proteins engineered from human components. J Clin Invest 119(10):3035-47.

Peden K, Emerman M, Montagnier L. 1991. Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1LAI, HIV-1MAL, and HIV-1ELI. Virology 185(2):661-72.

Perez EE, Wang J, Miller JC, Jouvenot Y, Kim KA, Liu O, Wang N, Lee G, Bartsevich VV, Lee YL, Guschin DY, Rupniewski I, Waite AJ, Carpenito C, Carroll RG, Orange JS, Urnov FD, Rebar EJ, Ando D, Gregory PD, Riley JL,

Holmes MC, June CH. 2008. Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. Nat Biotechnol 26(7):808-16.

Peterlin BM, Trono D. 2003. Hide, shield and strike back: how HIV-infected cells avoid immune eradication. Nature Reviews Immunology 3, 97-107.

Pham QT, Bouchard A, Grütter MG, Berthoux L. 2010. Generation of human TRIM5alpha mutants with high HIV-1 restriction activity. Gene Ther 17(7):859-71.

Pluta K, Kacprzak MM. 2009. Use of HIV as a gene transfer vector. ActaBiochim Pol 56(4):531-95.

Qin XF, An DS, Chen IS, Baltimore D. 2003. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc Natl Acad Sci U S A 100(1):183-8.

Quillent C, Oberlin E, Braun J, Rousset D, Gonzalez-Canali G, Métais P, Montagnier L, Virelizier JL, Arenzana-Seisdedos F, Beretta A. 1998. HIV-1-resistance phenotype conferred by combination of two separate inherited mutations of CCR5 gene. Lancet 351(9095):14-8.

Rambaut A, Posada D, Crandall KA, Holmes EC. 2004. The causes and consequences of HIV evolution. Nat Rev Genet 5(1):52-61.

Rangel HR, Garzaro D, Rodríguez AK, Ramírez AH, Ameli G, Del Rosario Gutiérrez C, Pujol FH. 2009. Deletion, insertion and stop codon mutations in vif genes of HIV-1 infecting slow progressor patients. J Infect Dev Ctries 3(7):531-8.

Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, Josephs SF, Doran ER, Rafalski JA, Whitehorn EA, Baumeister K, et al. 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 313(6000):277-84.

Reynes J, Portales P, Segondy M, Baillat V, André P, Avinens O, Picot MC, Clot J, Eliaou JF, Corbeau P. 2001. CD4 T cell surface CCR5 density as a host factor in HIV-1 disease progression. AIDS 15(13):1627-34.

Rho HM, Poiesz B, Ruscetti FW, Gallo RC. 1981. Characterization of the reverse transcriptase from a new retrovirus (HTLV) produced by a human cutaneous T-cell lymphoma cell line. Virology 112(1):355-60.

Ringpis GE, Shimizu S, Arokium H, Camba-Colón J, Carroll MV, Cortado R, Xie Y, Kim PY, Sahakyan A, Lowe EL, Narukawa M, Kandarian FN, Burke BP,

Symonds GP, An DS, Chen IS, Kamata M. 2012. Engineering HIV-1-resistant T-cells from short-hairpin RNA-expressing hematopoietic stem/progenitor cells in humanized BLT mice. PLoS One 7(12):e53492.

Rossi JJ, June CH, Kohn DB. 2007. Genetic therapies against HIV. Nat Biotechnol 25(12):1444-54.

Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Rooney DL, Zhang M, Ihrig MM, McManus MT, Gertler FB, Scott ML, Van Parijs L. 2003. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet 33(3):401-6.

Saayman S, Barichievy S, Capovilla A, Morris KV, Arbuthnot P, Weinberg MS. 2008. The efficacy of generating three independent anti-HIV-1 siRNAs from a single U6 RNA Pol III-expressed long hairpin RNA. PLoS One 3(7):e2602.

Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman RG, Doms RW, Vassart G, Parmentier M. 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature 382(6593):722-5.

Sano M, Li H, Nakanishi M, Rossi JJ. 2008. Expression of long anti-HIV-1 hairpin RNAs for the generation of multiple siRNAs: advantages and limitations. MolTher 16(1):170-7.

Sayah DM, Sokolskaja E, Berthoux L, Luban J. 2004. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. Nature 430(6999):569-73.

Scherer LJ, Rossi JJ. 2011. Ex vivo gene therapy for HIV-1 treatment. Hum Mol Genet 20(R1):R100-7.

Schopman NC, Liu YP, Konstantinova P, ter Brake O, Berkhout B. 2010. Optimization of shRNA inhibitors by variation of the terminal loop sequence. Antiviral Res 86(2):204-11.

Sharma A, Larue RC, Plumb MR, Malani N, Male F, Slaughter A, Kessl JJ, Shkriabai N, Coward E, Aiyer SS, Green PL, Wu L, Roth MJ, Bushman FD, Kvaratskhelia M. 2013. BET proteins promote efficient murine leukemia virus

integration at transcription start sites. Proc Natl Acad Sci U S A110(29):12036-41.

Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. 2003. Activation of the interferon system by short-interfering RNAs. Nat Cell Biol5(9):834-9.

Snøve O Jr, Rossi JJ. 2006. Expressing short hairpin RNAs in vivo. Nat Methods 3(9):689-95.

Sodroski JG, Rosen CA, Haseltine WA. 1984. Trans-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. Science 225(4660):381-5.

Steckbeck JD, Kuhlmann AS, Montelaro RC. 2013. C-terminal tail of human immunodeficiency virus gp41: functionally rich and structurally enigmatic. J Gen Virol 94(Pt 1):1-19.

Stevenson M. 2013. CROI 2013: Basic science review. Top Antivir Med21(2):42-6.

Strack B, Calistri A, Accola MA, Palu G, Gottlinger HG. 2000. A role for ubiquitin ligase recruitment in retrovirus release. Proc Natl Acad Sci U S A 97(24):13063-8.

Strack B, Calistri A, Göttlinger HG. 2002. Late assembly domain function can exhibit context dependence and involves ubiquitin residues implicated in endocytosis. J Virol 76(11):5472-9.

Strack B, Calistri A, Craig S, Popova E, Göttlinger HG. 2003. AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. Cell 114(6):689-99.

Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J. 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature 427(6977):848-53.

Tebas P, Stein D, Binder-Scholl G, Mukherjee R, Brady T, Rebello T, Humeau L, Kalos M, Papasavvas E, Montaner LJ, Schullery D, Shaheen F, Brennan AL, Zheng Z, Cotte J, Slepushkin V, Veloso E, Mackley A, Hwang WT, Aberra F, Zhan J, Boyer J, Collman RG, Bushman FD, Levine BL, June CH. 2013. Antiviral effects of autologous CD4 T cells genetically modified with a conditionally replicating lentiviral vector expressing long antisense to HIV. Blood 121(9):1524-33.

ter Brake O, Konstantinova P, Ceylan M, Berkhout B. 2006. Silencing of HIV-1 with RNA interference: a multiple shRNA approach. Mol Ther 14(6):883-92.

ter Brake O, 't Hooft K, Liu YP, Centlivre M, von Eije KJ, Berkhout B. 2008. Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. MolTher 16(3):557-64.

Terwilliger EF, Godin B, Sodroski JG, Haseltine WA. 1989. Construction and use of a replication-competent human immunodeficiency virus (HIV-1) that expresses the chloramphenicol acetyltransferase enzyme. Proc Natl Acad Sci U S A 86(10): 3857–3861.

Terwilliger EF, Sodroski JG, Haseltine WA. 1990. Mechanisms of infectivity and replication of HIV-1 and implications for therapy. Ann Emerg Med 19(3):233-41.

Theodore TS, Englund G, Buckler-White A, Buckler CE, Martin MA, Peden KW. 1996. Construction and characterization of a stable full-length macrophage-tropic HIV type 1 molecular clone that directs the production of high titers of progeny virions. AIDS Res Hum Retroviruses 12(3):191-4.

Tyagi A, Ahmed F, Thakur N, Sharma A, Raghava GP, Kumar M. 2011. HIVsirDB: a database of HIV inhibiting siRNAs. PLoS One 6(10):e25917.

Vergis EN, Mellors JW. 2000. Natural history of HIV-1 infection. Infect Dis Clin North Am14(4):809-25, v-vi.

Walker JE, Chen RX, McGee J, Nacey C, Pollard RB, Abedi M, Bauer G, Nolta JA, Anderson JS. 2012. Generation of an HIV-1-resistant immune system with CD34(+) hematopoietic stem cells transduced with a triple-combination anti-HIV lentiviral vector. J Virol86(10):5719-29.

Whitcomb JM, Huang W, Fransen S, Limoli K, Toma J, Wrin T, Chappey C, Kiss LD, Paxinos EE, Petropoulos CJ. 2007. Development and characterization of a novel single-cycle recombinant-virus assay to determine human immunodeficiency virus type 1 coreceptor tropism. Antimicrob Agents Chemother 51(2):566-75.

Wilen CB, Wang J, Tilton JC, Miller JC, Kim KA, Rebar EJ, Sherrill-Mix SA, Patro SC, Secreto AJ, Jordan AP, Lee G, Kahn J, Aye PP, Bunnell BA, Lackner AA, Hoxie JA, Danet-Desnoyers GA, Bushman FD, Riley JL, Gregory PD, June CH, Holmes MC, Doms RW. 2011. Engineering HIV-resistant human CD4+ T cells with CXCR4-specific zinc-finger nucleases. PLoSPathog 7(4):e1002020.

Wiznerowicz M, Trono D. 2003. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. J Virol 77(16):8957-61.

Wu X, Li Y, Crise B, Burgess SM. 2003. Transcription start regions in the human genome are favored targets for MLV integration. Science 300 :1749–1751.

Yi R, Doehle BP, Qin Y, Macara IG, Cullen BR. 2005. Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. RNA11(2):220-6.

Yu JY, DeRuiter SL, Turner DL. 2002. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. ProcNatlAcadSci U S A 99(9):6047-52.

Zhou J, Rossi JJ. 2011. Current progress in the development of RNAi-based therapeutics for HIV-1. Gene Ther 18(12):1134-8.

Zhou S, Mody D, DeRavin SS, Hauer J, Lu T, Ma Z, Hacein-Bey Abina S, Gray JT, Greene MR, Cavazzana-Calvo M, Malech HL, Sorrentino BP. 2010. A self-inactivating lentiviral vector for SCID-X1 gene therapy that does not activate LMO2 expression in human T cells. Blood 116(6):900-8.

RINGRAZIAMENTI

Ringrazio sentitamente la Prof.ssa Cristina Parolin per avermi coinvolta in questo progetto di ricerca, per la supervisione e la discussione critica dei dati.
Ringrazio la Dott.ssa Arianna Calistri per il supporto scientifico e la Dott.ssa Claudia Del Vecchio per il lavoro svolto insieme nel primo periodo di Dottorato.
Desidero ringraziare il Prof. Giorgio Palù, per avermi dato l'opportunità di svolgere il Dottorato presso il Dipartimento di Medicina Molecolare e la Prof.ssa Marina Cavazzana per avermi ospitata nel Suo laboratorio.
Ringrazio il Prof. Saverio Parisi per la collaborazione al progetto di ricerca.
Ringrazio i miei colleghi, in particolare il Dott. Michele Celestino e le Dott.sse Ilaria Carli, Barbara Mantelli, Valentina Poletti e Marta Toffoletto, per la loro collaborazione ed il loro supporto. Un grazie di cuore alla Dott.ssa Anna D'Angelillo, per l'affettuoso aiuto quotidiano in laboratorio.
Grazie, infine, a tutte le persone che con il loro affetto e la loro amicizia mi sono state vicine in questi tre anni.