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GUIDELINES FOR BASIC SCIENCE

Selection of RNAi-based inhibitors for anti-HIV gene therapy

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Abstract

In the last decade, RNA interference (RNAi) advanced to one of the most widely applied techniques in the biomedical research field and several RNAi therapeutic clinical trials have been launched. We focus on RNAi-based inhibitors against the chronic infection with human immunodeficiency virus type 1 (HIV-1). A lentiviral gene therapy is proposed for HIV-infected patients that will protect and reconstitute the vital immune cell pool. The RNAi-based inhibitors that have been developed are short hairpin RNA molecules (shRNAs), of which multiple are needed to prevent viral escape. In ten distinct steps, we describe the selection process that started with 135 shRNA candidates, from the initial design criteria, *via* testing of the *in vitro* and *in vivo* antiviral activity and cytotoxicity to the final design of a combinatorial therapy with three shRNAs. These shRNAs satisfied all 10 selection criteria such as targeting conserved regions of the HIV-1 RNA genome, exhibiting robust inhibition of HIV-1 replication and having no impact on cell physiology. This combinatorial shRNA vector will soon move forward to the first clinical studies.

Key words: Human immunodeficiency virus type 1; RNA interference; Gene therapy; "Human Immune System" mouse; Lentivirus

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INTRODUCTION

After discovery of the mechanism of RNA interference (RNAi) in C. elegans in 1998[1], several RNAi approaches have been developed for use in therapeutic strategies, e.g., against inherited diseases or infectious pathogens[2]. The cellular RNAi pathway leads to the processing of small noncoding microRNAs (miRNAs) that regulate cellular gene expression at the post-transcriptional level to control cell differentiation and development[3]. This pathway may be primed by artificial short hairpin RNAs (shRNAs) that are produced in the cell from a transgene and processed into small interfering RNAs (siRNAs)[4]. Ready-to-use siRNAs can also be synthesized chemically and transfected into cells. Perfect base-pairing of the designed siRNA with the specific mRNA target results in cleavage of the latter by the RNA-induced silencing complex (RISC)[5]. Topical delivery of siRNAs in the lungs might be feasible for the treatment of acute infections with e.g.,

influenza A virus or the respiratory syncytial virus. However, chronic infections caused by pathogens such as the human immunodeficiency virus type 1 (HIV-1), hepatitis B virus and hepatitis C virus will require the continuous expression of RNAi inhibitors from a therapeutic transgene.

HIV-1 is replicating in cells of the human immune system, resulting in a constant depletion of the CD4+ T cells that contributes to the eventual progression to AIDS. Anti-HIV gene therapy aims to protect this indispensable cell pool from virus infection and destruction, which should lead to a (partial) reconstitution of the immune system. Due to the chronic nature of HIV-1 infection, cells must be protected life-long against HIV-1, which can be achieved by a stable RNAi gene therapy against the HIV-1 RNA genome. Apart from RNAi approaches, other antiviral strategies can be utilized such as ribozymes, antisense RNAs, dominant negative protein variants, decoy RNAs, or combinations of RNAi, ribozymes and RNA decoys[6]. However, the simultaneous use of multiple RNAi inhibitors seems one of the most promising approaches for a potent and durable therapy[7]. The therapeutic protocol that we have in mind starts with the isolation of blood mobilized CD34+ hematopoietic progenitor cells from HIV-infected patients, followed by *ex vivo* transduction with an shRNA-expressing lentiviral vector that stably integrates in the host cell DNA, and re-injection of the modified cells into the patients. Target cells for HIV-1 infection (CD4+ T cells, monocytes/macrophages and dendritic cells) that originate from these transduced pluripotent progenitor cells will express the antiviral RNAi constructs and thus prevent HIV-1 gene expression and virus replication. By that, HIV-1 infected CD4+ T cells evade also the destruction by CD8+ cytotoxic T cells as HIV-1 protein production can trigger viral peptide presentation *via* the MHC class I molecules to cytotoxic T cells.

We and others have previously demonstrated remarkably potent virus inhibition even with a single shRNA, but also observed that HIV-1 quickly escapes from RNAi pressure *via* the selection of mutations in the targeted sequence[8-13]. However, a combination of multiple potent shRNAs provided long-term suppression of HIV-1 replication[14-16]. For several years, we have designed and tested various alternative RNAi strategies against HIV-1. Extended shRNA designs and miRNA-like polycistron transcripts were optimized for the expression of multiple inhibitors, but the use of independent shRNA cassettes turned out to be most efficient[9,14,17-21]. Thus, the goal is to use a lentiviral vector with multiple shRNA cassettes that becomes stably incorporated in the human genome. We therefore designed a battery of shRNA inhibitors and tested these in a variety of *in vitro* and *in vivo* experimental settings to allow the selection of the most potent and safe RNAi antivirals. The top candidates were subsequently chosen for the development of a combinatorial RNAi gene therapy against HIV-1 that will be translated into a clinical trial[16]. Primary safety and efficacy studies were performed in the "Human Immune System" (HIS) mouse model[22,23]. Human CD34+ hematopoietic progenitor cells (hHPC) were transduced *ex vivo* with the lentiviral RNAi expression constructs and injected into immunocompromised newborn mice to monitor cell development and differentiation, shRNA expression, cytotoxicity and efficacy of the therapeutic regimen upon HIV-1 infection[24]. This pre-clinical animal model does closely mimic the anti-HIV gene therapy approach proposed for HIV-infected patients.

Here, we will discuss the numerous criteria and corresponding experimental tests that were used in selecting the optimal shRNA reagents for a combinatorial attack on the HIV-1 RNA genome. Ten distinct selection steps can be envisaged (Figure 1): (1) the basic design of the shRNA gene cassettes; (2 + 3) measurement of the antiviral activity in transiently transfected cells and stably transduced cells; (4) selection of the most conserved HIV-1 target sequences to maximize the number of sensitive viral isolates; (5) testing the viral escape possibilities as a measure of the durability of the therapeutic attack; (6) criteria imposed by the use of a lentiviral vector for delivery of the antiviral shRNA cassettes; (7 + 8) screens for possible adverse effects on cell physiology, both *in vitro* and *in vivo*; (9) target site alterations due to resistance mutations for clinically approved antiretroviral drugs; and (10) the assembly of multiple shRNAs to establish a combinatorial RNAi therapy. Along this selection pathway, which took over 7 years, we tested more than 135 shRNA candidates to end up with three potent and safe shRNAs that will be employed in a gene therapy trial (Table 1).

DESIGN OF shRNA MOLECULES

To identify new and potent shRNAs against HIV-1, different design criteria were applied. In general, the shRNA design was based on the prototype shRNA hairpin transcript published by Brummelkamp in 2002: complementary 19-nucleotide sense and antisense strands, a 9-nucleotide hairpin loop and 3'-UU overhang[4]. The antisense strand of this shRNA design will, upon Dicer processing, form the guide strand that instructs RISC for antiviral attack. The complete shRNA cassette consists of the RNA polymerase III H1 promoter, the shRNA sequence followed by the TTTTT termination signal. The H1 promoter, shRNA and termination signal were designed as synthetic DNA or as restriction fragments and cloned into the pSUPER vector (Figure 2A). This cassette can easily be transferred into the lentiviral vector JS1 (Figure 2B) for generation of stably transduced cells[25]. All shRNAs were checked *in silico* to avoid significant complementarity against cellular mRNAs to prevent putative off-target effects.

Over the years, several sets of shRNA inhibitors were tested in our laboratory. We initially described potent suppression of HIV-1 replication with an shRNA that targets nef gene sequences, but viral escape was appar-

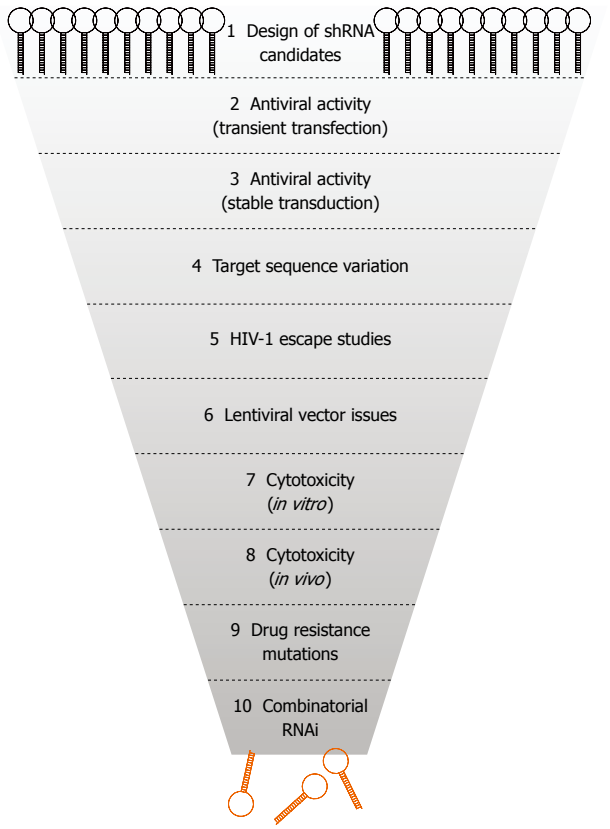


Figure 1 Selection of short hairpin RNAs against human immunodeficiency virus type 1. Scheme of the 10 steps used for the selection of the best shRNA inhibitors for development of an antiviral RNA interference (RNAi) gene therapy. HIV-1: Human immunodeficiency virus type 1.

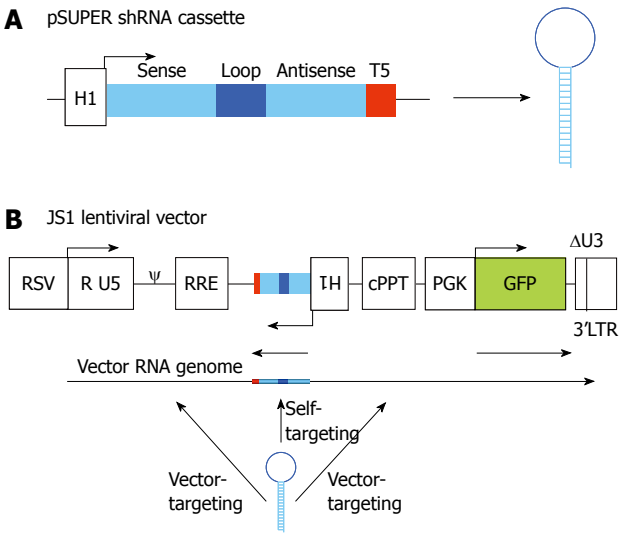


Figure 2 RNA interference vector constructs. A: Two complementary DNA oligonucleotides are annealed and cloned into pSUPER downstream of the H1 promoter that triggers short hairpin RNA (shRNA) expression. The shRNA cassette encodes a 19 nt sense strand, 9 nt loop, 19 nt antisense strand and a stretch of 5 T's (T5), which is the termination signal; B: The shRNA cassette was cloned in the lentiviral vector JS1 for stable transduction of human T cells. The shRNA cassette is cloned in antisense direction to avoid promoter interference. During vector production three transcripts are produced from the lentiviral vector: the shRNA, the vector RNA genome and the GFP transcript. The shRNA will have a 100% target match with the shRNA-encoding sequence in the vector RNA genome (self-targeting), and potential targets in the human immunodeficiency virus type 1 (HIV-1) derived sequences of the lentiviral vector (vector-targeting). RSV: Respiratorial syncytial virus promoter; R U5: R and U5 element of HIV-1 promoter; ψ: Packaging signal; RRE: Rev responsive element; cPPT: Central polypurine tract; PGK GFP: Green fluorescent protein driven from a PGK promoter; 3'LTR: 3' long terminal repeat of HIV-1, with deletion in the U3 region.

ent in prolonged cultures[9,13]. We next tested a first set of 86 antiviral shRNAs that were selected based solely on the conservedness of the target sequence among HIV-1 isolates[14]. Due to the high variability of HIV-1, this selection criterion has become very important for the development of a gene therapy that applies to a broad range of isolates. Our initial studies also revealed the importance of taking the target RNA structure into account for shRNA design as occluded targets are poorly recognized by the RNAi machinery[13,26]. Therefore, we generated a second set of shRNAs that targets particularly accessible regions of the HIV-1 RNA genome based on the SHAPE determined RNA structure model[27,28].

ANTIVIRAL ACTIVITY IN TRANSIENTLY TRANSFECTED CELLS

To evaluate the potency of the shRNAs in terms of anti-HIV activity, we developed a test to measure the inhibition of HIV-1 protein production[14]. For that reason, 293T cells were co-transfected with the HIV-1 molecular clone pLAI, the pSUPER-shRNA vector and the pRL Renilla vector to control for the transfection efficiency. These transfected 293T cells produce infectious virus but do not allow new rounds of infection due to the absence of relevant receptors for HIV-1 attachment and entry. At 48 h post-transfection, the HIV-1 capsid protein (CA-p24) production and Renilla production were quantified. CA-p24 can easily be measured *via* CA-p24 ELISA in the culture supernatant. Then, CA-p24 levels normalized for Renilla expression were compared to virus production with the empty pSUPER control plasmid obtained in co-transfections[14]. Figure 3 indicates the target sites for the most potent RNAi inhibitors plotted onto the HIV-1 genome. Of the 135 shRNA candidates, 44 exhibited at least 80% suppression of HIV-1 production. The upper panel depicts the first set, the lower panel marks the target sites for the second shRNA set[14,27]. Table 1 summarizes the characteristics of the 44 shRNAs that exhibit robust inhibition.

ANTIVIRAL ACTIVITY IN STABLY TRANSDUCED T CELLS

Several of the shRNAs that exhibited significant antiviral activity in the transient transfection assay were subsequently tested in stably transduced CD4+ T cells. To do so, the shRNA expression cassettes were cloned into the lentiviral vector JS1 to allow stable transduction of SupT1 T cells (Figure 2B)[11,14,25]. SupT1 is a commonly used CD4+ T cell line that is permissive for HIV-1 infec-

Table 1 Selection of short hairpin RNAs against human immunodeficiency virus type 1

shRNA name	HIV-1 target		HIV-1 inhibition		Target conservation (%)[5]		Viral escape[6]	Lentiviral vector		Cell toxicity[9]		Drug resistance mutation[10]
	Position[1]	Gene	Transient[3]	Stable[4]	Subtype B	All subtypes		Vector targeting[7]	Titer reduction[8]	*In vitro*	*In vivo*	
LDR2	327[2]	Leader	95	++	66	61	ND	+	ND	ND	ND	-
LDR3	328[2]	Leader	94	++	68	61	ND	+	ND	ND	ND	-
LDR4	329[2]	Leader	99	+++	68	61	ND	+	ND	ND	ND	-
LDR5	330[2]	Leader	94	++	68	61	ND	+	ND	ND	ND	-
LDR7	332[2]	Leader	84	-	69	61	ND	+	ND	ND	ND	-
LDR8	333[2]	Leader	79	++	69	61	ND	+	ND	ND	ND	-
LDR9	334[2]	Leader	91	+++	70	63	ND	+	ND	ND	ND	-
CA	1032	CA-p24	97	-	87	67	ND	-	-	-	ND	-
Gag5	1365	CA-p24	86	++	81	80	+	-	+	+	+	-
Pol1[11]	1910[2]	Prot[11]	97[11]	+++[11]	89[11]	85[11]	+[11]	-[11]	-[11]	-[11]	-[11]	D30N, V32I[11]
Pol2	1911[2]	Prot	86	-	89	85	ND	-	ND	ND	ND	D30N, V32I
Pro 1	1912[2]	Prot	99	+++	85	81	ND	-	-	-	ND	D30N, V32I
Pro 2	1913[2]	Prot	99	ND	80	79	ND	-	ND	ND	ND	D30N, V32I
Pro 3	1914[2]	Prot	99	ND	80	79	ND	-	ND	ND	ND	D30N, V32I
Pro 4	1915[2]	Prot	98	ND	77	76	ND	-	ND	ND	ND	D30N, V32I, L33F
Pro 5	1916[2]	Prot	97	ND	79	78	ND	-	ND	ND	ND	D30N, V32I, L33F
Pro 6	1918[2]	Prot	98	ND	78	77	ND	-	ND	ND	ND	D30N, V32I, L33F
Pro 7	1919[2]	Prot	98	ND	77	76	ND	-	ND	ND	ND	D30N, V32I, L33F
Pro 8	2026	RT	87	ND	58	17	ND	-	ND	ND	ND	D30N, V32I, L33F
Pol6	3755[2]	RT	97	-	72	75	ND	-	ND	ND	ND	-
RT 1 (A)	3757[2]	RT	95	++	71	74	ND	-	-	-	ND	-
RT 2 (B)	3758[2]	RT	98	++	73	75	ND	-	-	+	ND	-
RT 3 (C)	3759[2]	RT	95	++	73	75	ND	-	-	-	ND	-
RT 4 (F)	3760[2]	RT	85	++	69	69	ND	-	ND	ND	ND	-
RT 5 (G)	3762[2]	RT	94	-	72	72	ND	-	-	-	ND	-
Int 1	4310	Int	91	ND	71	19	ND	-	ND	ND	ND	-
Int 2	4344	Int	96	+++	67	25	ND	+	+	-	ND	-
Pol29	4393	Int	82	-	80	80	ND	+	ND	ND	ND	-
Int 3	4420[2]	Int	95	+++	68	61	ND	+	+	+	ND	-
Int 4	4422[2]	Int	99	ND	71	64	ND	+	ND	ND	ND	-
Int 5	4491	Int	81	ND	70	33	ND	-	ND	ND	ND	-
Pol45	4543[2]	Int	92	++	91	75	ND	-	ND	ND	ND	-
Pol47[11]	4545[2]	Int[11]	92[11]	+++[11]	91[11]	73[11]	+[11]	-[11]	-[11]	-[11]	-[11]	-[11]
Vif	4646	Int/Vif	96	-	82	31	ND	-	-	-	ND	-
R/T5[11]	5551	Rev/Tat[11]	93[11]	+++[11]	87[11]	73[11]	+[11]	-[11]	-[11]	-[11]	-[11]	-[11]
Env 1	7250	gp120	81	ND	83	76	ND	+	ND	ND	ND	-
Env 2	7875[2]	gp41	83	ND	49	33	ND	-	ND	ND	ND	-
Env 3	7881[2]	gp41	89	ND	19	6	ND	+	ND	ND	ND	-
Env 4	7884[2]	gp41	85	ND	21	6	ND	+	ND	ND	ND	-
Env 5	8026	gp41	96	+++	53	14	ND	+	+	+	ND	-
Env 6	8277[2]	gp41	98	++	53	18	ND	-	-	+	ND	-
Env 7	8278[2]	gp41	98	++	56	18	ND	-	-	-	ND	-
Env 8	8359	gp41	97	+++	3	1	ND	-	-	-	ND	-
LTR	9072	3′LTR	95	++	53	1	ND	-	-	-	ND	-

[1]Position in human immunodeficiency virus type 1 (HIV-1) LAI mRNA; [2]Overlapping short hairpin RNA (shRNA) clusters; [3]Percentage of inhibition of HIV-1 production in co-transfected cells; [4]Inhibition of HIV-1 replication in stably transduced cells: +++ = strong, ++ = medium, + = low, - = no inhibition; [5]Percentage of sequences in Los Alamos database identical to shRNA target sequence; [6]Detection of escape mutations after prolonged culturing; [7]100% complementarity of the shRNA to JS1 lentiviral vector; [8]Titers compared to JS1 lentiviral vector; + = reduction > 1 log, - = reduction < 1 log; [9]Effects on cell growth; + = negative effect, - = no effect; [10]Drug resistance mutations in the shRNA target region; [11]Selected for the combinatorial gene therapy. ND: Not determined.

tion and that shows clear cytopathic effects (syncytia) upon virus replication. The cells were transduced at a low multiplicity of infection of 0.15 to assure that maximally a single copy of the lentiviral vector integrates per cell. The expression of a GFP reporter gene by the JS1 lentiviral vector allows the easy separation of transduced from non-transduced cells by fluorescence activated cell sorting. The cells are usually sorted 2 d after transduction to obtain a pure GFP-positive population. The transduced cells can subsequently be challenged with HIV-1 and virus replication can be monitored. Infected cultures were inspected on a daily basis under the microscope to monitor cytopathic effects and supernatants were collected to measure CA-p24 production (Figure 4A). SupT1 cells transduced with the empty JS1 vector served as control cells to measure uninhibited viral spread. For future gene therapy applications, shRNAs were only considered if they conferred strong HIV-1 inhibition in transient co-

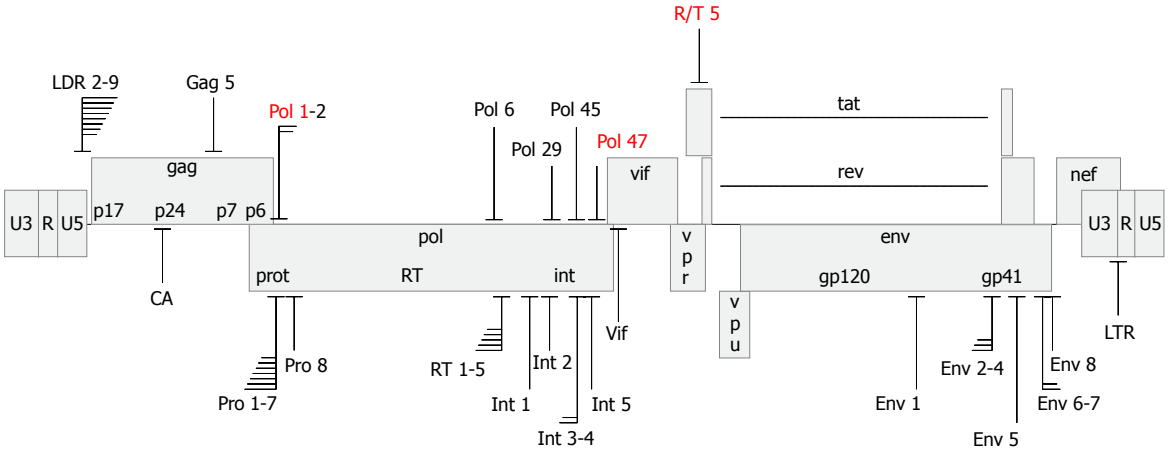


Figure 3 Target sites of anti-human immunodeficiency virus short hairpin RNAs. Depicted is the human immunodeficiency virus type 1 (HIV-1) LAI proviral genome with short hairpin RNA (shRNA) target sites that yielded > 80% suppression of HIV-1 production. Several clusters of overlapping shRNA targets are indicated. The shRNAs have been designed based on conservedness of the target sequence (upper panel) or accessibility in the structured HIV-1 RNA genome (lower panel). shRNAs selected for the R3 combinatorial RNA interference vector are marked in red.

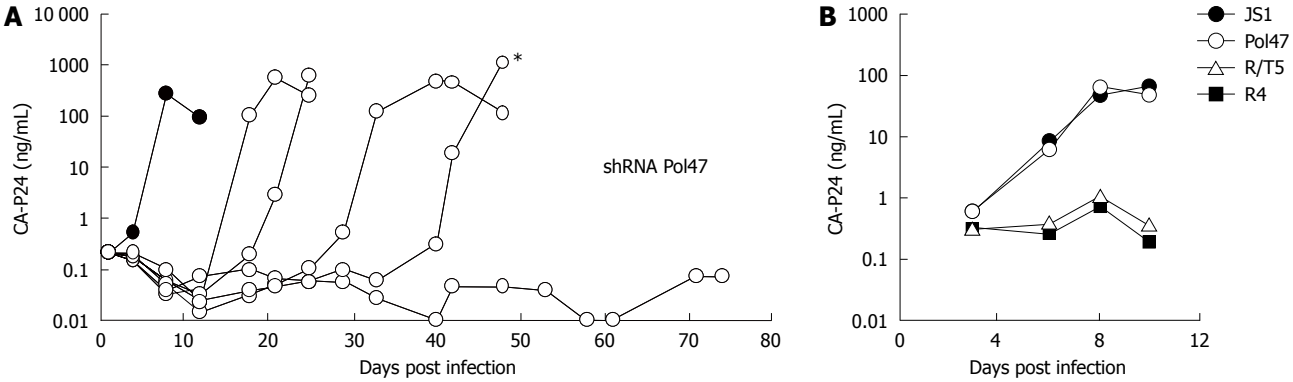
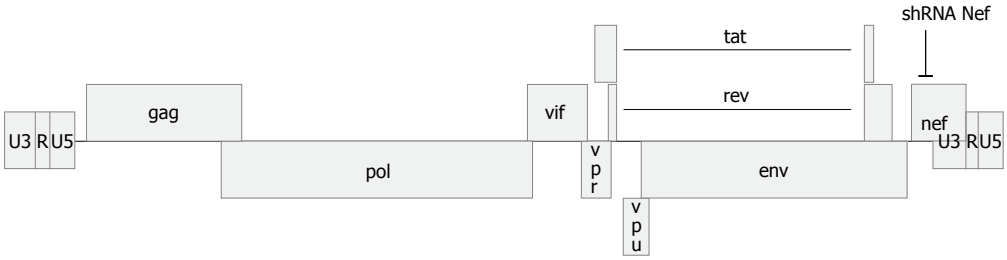


Figure 4 Long-term short hairpin RNA activity in human immunodeficiency virus type 1 replication studies. A: SupT1 T cells expressing short hairpin RNA (shRNA) Pol47 were infected with HIV-1 and virus replication was monitored in five cultures by measuring CA-p24 production for up to 75 d. Cells transduced with the empty lentiviral vector JS1 served as control (dark circles); B: Supernatant from the indicated culture (asterisk in panel A) was passaged on new cells to test the escape phenotype. The virus replicated on control and shRNA Pol47 cells, but not on cells that express another antiviral shRNA (R/T5) or the R4 construct. Adapted from[16].

transfections and stably transduced T cells. In Table 1, this is indicated as "+++" in the respective column.

HIV-1 TARGET SEQUENCE VARIABILITY

Due to the high variability of HIV-1, it is especially important for an anti-HIV gene therapy to target sequences that are relatively conserved among different virus isolates and several HIV-1 subtypes. For most shRNAs, this was an important selection criterion and Table 1 provides an overview of the conservedness of the shRNA targets, both for subtype B that is most prevalent in the Western world and the other subtypes that belong to the HIV-1 group M. To determine the degree of conservedness of the siRNA target sequences, all HIV-1 genome sequences present in the Los Alamos National Laboratory database (http://www.hiv.lanl.gov/) were aligned. The alignment provides the percentage of sequences that are fully complementary to the siRNA for the entire group M and also per subtype. To ensure high sequence conservation, minimally 70% of the viral sequences of each target region have to form a perfect match with the siRNA[14]. This was important for the group M sequences that comprise all subtypes, and specifically the subtype B sequences that are most prevalent in the Western world (Table 1). This standard ensures targeting of a broad spectrum of viral isolates and also decreases the risk of rapid viral escape because mutations of well-conserved HIV-1 sequences are more likely to cause a loss of viral replication efficiency[9-11,13,29,30]. As a current standard diagnostic procedure, the patient-derived HIV-1 sequences of the pol gene are genotyped, including the target sequences for the shRNA inhibitors Pol1 and Pol47. Thus, one will be able to confirm the conservation, such that a full match with the shRNA is guaranteed. Genotyping will also reveal the presence of non-B subtypes, exotic HIV-1 strains and even super-infections that may complicate the RNAi gene therapy[31].



Cultures	Sampling day	shNef target 164 182	
wt		GCTGCTTGTGCCTGGCTAGAAGCACAAG	
A	43	----------	106-nt deletion
B	46	GCTGCTTGT----------AGCACAAG	
C1	27	GCTGCTTGTGCCTGGCGAGAAGCACAAG	
C2	62	GCTGCTTGTACCTGGCGAGAAGCACAAG	
D	62	GCTGCT------TGGCTAGAAGCACAAG	
E	62	GCTGCTTGTGCCTGGCTAGAAG------	63-nt deletion
F	80	GCTGCTTGTGCCTGGCTAGAGGCACAAG	
G	77	----------	225-nt deletion
H	74	ACTGCTTGTGCCTGGCTAGAAGCACAAG	
I	61	GCTGCTTGTGCCTGGATAGAAGCACAAG	

Figure 5 Human immunodeficiency virus type 1 escapes from short hairpin RNA Nef. The human immunodeficiency virus type 1 (HIV-1) LAI proviral genome and the short hairpin RNA (shRNA) Nef target site are indicated. SupT1 cells expressing shRNA Nef were infected with HIV-1 and passaged twice a week until viral escape occurred. Nine different cultures were examined in parallel and the day of sampling is indicated. Part of the nef gene is shown with the shRNA Nef target site highlighted in gray. Numbers refer to the nucleotide position in the nef gene. Escape was apparent by (1) one or more escape mutations in the target sequence; (2) mutations outside the target region; and (3) complete or partial deletions of the target region. Mutations are underlined. Adapted from[13].

HIV-1 ESCAPE STUDIES

Viral escape from the shRNA pressure can occur similar to what is observed under antiviral therapy with antiviral drugs when the HIV-1 target sequence accumulates one or multiple mutations. Extensive viral escape studies have been performed for some shRNAs[8-11,13,32-34]. Transduced and GFP-sorted SupT1 cells were challenged with a high amount of virus and passaged over time. When viral outgrowth was observed, the cell-free supernatant was transferred to a new culture of shRNA-expressing cells to confirm the resistance phenotype (Figure 4A and B). Cellular DNA with the integrated proviral genome can subsequently be isolated, the siRNA target region can be PCR-amplified and cloned into a plasmid for sequence analysis[11,13,16,35]. At this point, it is important to filter out 'pseudo-escape' events that are due to breakthrough virus replication when a high virus input is tested, often in combination with a sub-optimal inhibitory shRNA regimen. In this scenario, the pseudo-escape virus can be recognized because it will not carry any resistance mutation and will obviously lack the resistance phenotype[9,11,16].

We routinely test multiple HIV-1 evolution cultures in parallel because viral evolution is a chance process driven by randomly occurring mutations, some of which are beneficial and thus subsequently selected under RNAi pressure. For example, the diverse viral escape routes observed in independent cultures of shRNA Nef expressing cells are depicted in Figure 5[13]. We reported three types of HIV-1 escape: (1) Mutation(s) in the siRNA target sequence; (2) a mutation in the flanking region that influences the local RNA structure; and (3) partial or even complete deletion of the target sequence. The latter escape route seems possible only in case non-essential viral sequences are targeted. Indeed, deletion-based viral escape was never witnessed when essential HIV-1 sequences encoding the Protease and Integrase enzymes were targeted[10,32]. This observation supports the notion to target well-conserved viral sequences that usually encode the more essential viral functions. It must be pointed out that viral escape studies are extremely time and labor-intensive. Therefore, such investigations should only be conducted for candidate shRNAs that fulfill multiple criteria, e.g., potent inhibition in transient transfections and stably transduced T cell infections.

LENTIVIRAL VECTOR CONSIDERATIONS

HIV-1 causes a persistent infection in humans, which requires durable expression of the inhibitory shRNAs. Therefore, the use of a lentiviral vector seems ideal because of its property to stably integrate into the host cell genome, which allows a constant supply of antiviral shRNAs. The third generation lentiviral vectors have proven to be safe for use in humans and no insertional oncogenesis has been reported thus far[36-38]. These vectors transduce dividing and non-dividing cells and can thus be applied, e.g., in hematopoietic progenitor cells[23,39-43]. For clinical application, it is important that the vector can be produced to high titers. We and others previously report-

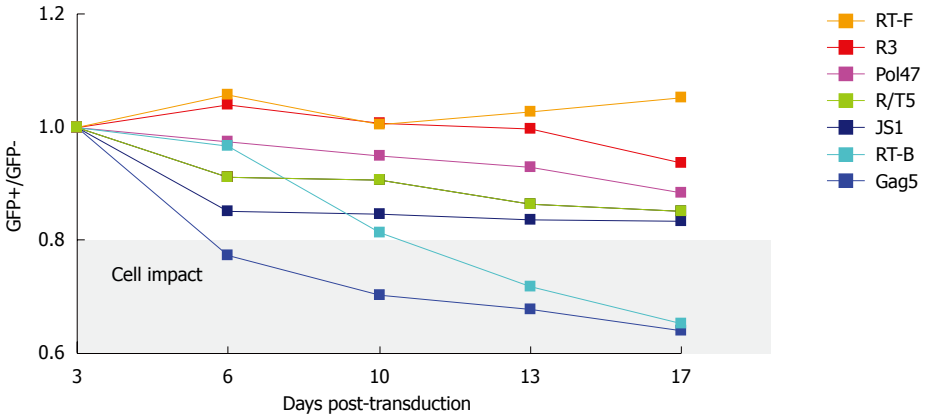


Figure 6 Competitive cell growth assay. SupT1 T cells were transduced with a short hairpin RNA (shRNA)-expressing lentiviral vector, yielding a cell population with approximately 40% GFP-positive cells. The ratio of GFP-positive cells at day 3 after transduction was set at 1 and measured longitudinally. The cells were passaged and analyzed *via* FACS measurement twice a week. JS1 represents the empty lentiviral vector without shRNA expression. The gray window highlights shRNAs that have a significant adverse effect on cell growth.

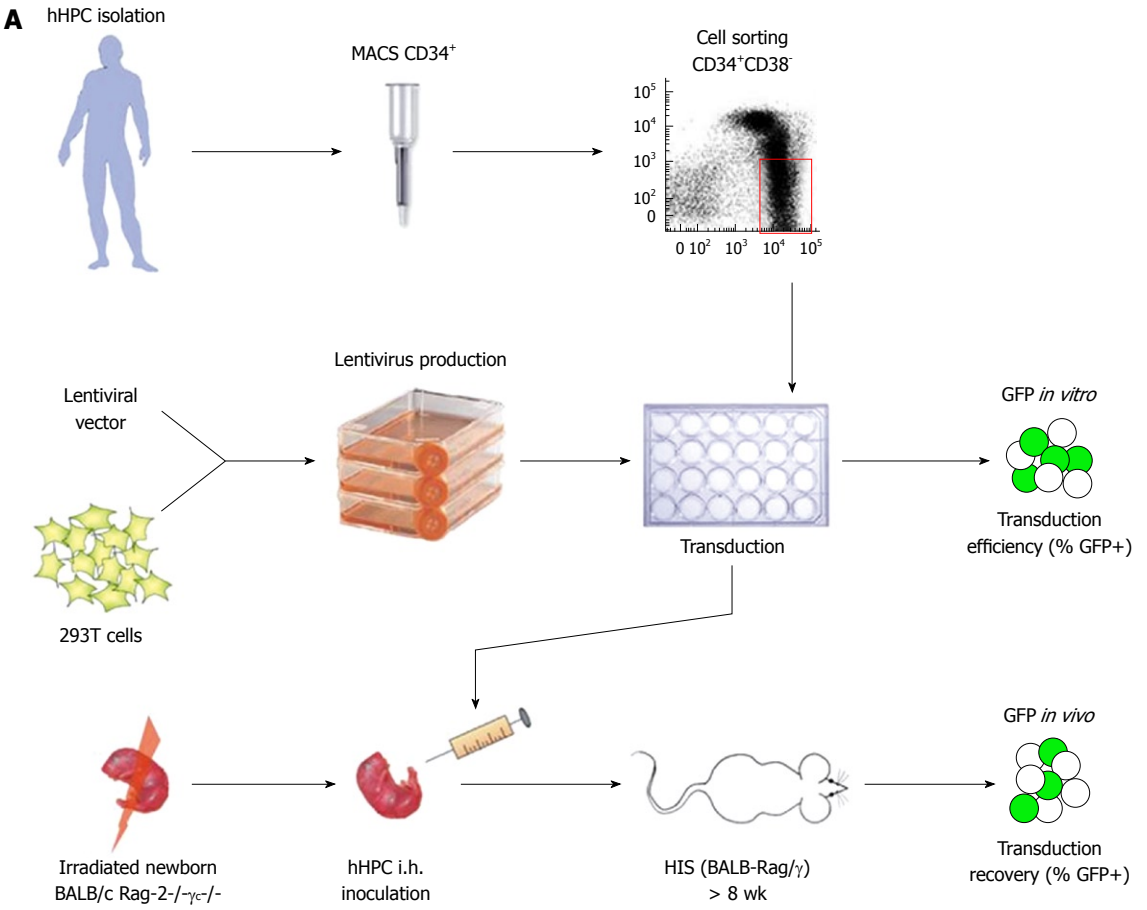
ed titer problems with lentiviral vectors encoding antiviral shRNAs that may obstruct clinical application[44-48]. Lentiviral vectors are produced by co-transfection of the lentiviral vector construct JS1 (Figure 2B), Gag-Pol and Rev-expression plasmids, and a VSV-G envelope construct. During lentiviral vector production, all the different mRNAs are expressed in the producer cell together with the shRNA transcript. The vector transcript does in fact include the shRNA sequence and will thus have a perfect target for siRNA-mediated degradation. However, such self-targeting does not easily occur because the target is occluded in a stable shRNA hairpin structure and therefore protected from RNAi attack. Further complications arise when the shRNA targets HIV-1 derived sequences in one of the lentiviral vector constructs. This is referred to as vector targeting. We previously discussed in detail all possible routes by which shRNAs could impede lentiviral vector production and how to prevent or overcome these specific problems[45,48]. Of course, acute cytotoxicity of the expressed shRNA can also cause a serious titer reduction due to effects on the producer cell viability and this may eventually also affect the viability of transduced cells, i.e., the gene therapy target cells.

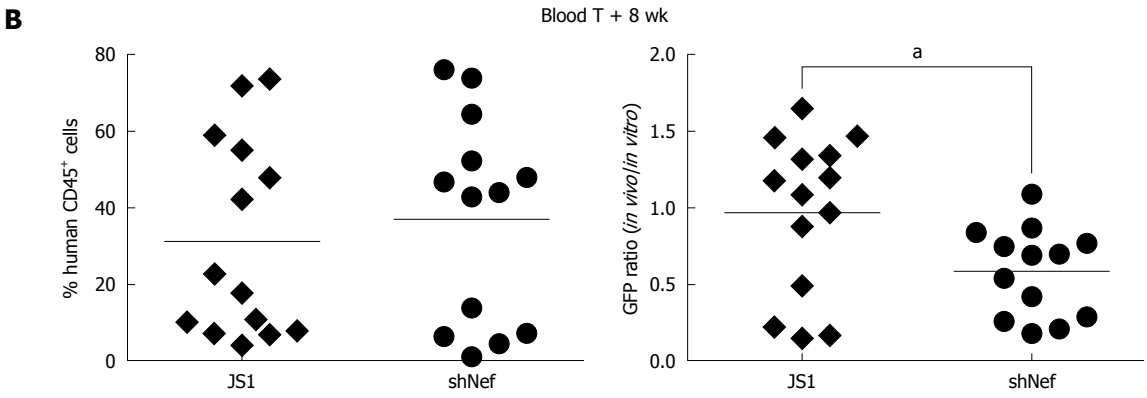
CYTOTOXICITY IN *IN VITRO* CELL CULTURE

The antiviral shRNA may exhibit adverse effects on cell growth through silencing of cellular mRNAs (off-target effects) or saturation of the RNAi machinery, in particular when the shRNA is overexpressed[49]. These effects cannot easily be predicted and should thus be tested experimentally. There are several ways to score the impact of shRNA expression on cell viability and physiology. One could for instance determine the cellular doubling time by frequent cell counting. We recently developed a very user-friendly and ultra-sensitive assay that follows over time the ratio of shRNA-expressing GFP-positive cells *vs* untransduced GFP-negative cells in a co-culture assay[50]. This competitive cell growth or CCG assay has some clear advantages over other well-established cell proliferation assays: (1) After cell transduction, only a small aliquot of the culture is needed to launch the CCG assay, without any extra steps; (2) The CCG assay is internally controlled as it starts with a mixture of transduced and untransduced cells; and (3) Even minor effects on the cellular proliferation rate caused by shRNA expression can be detected. We screened all promising shRNAs in this assay (Figure 6). Besides single shRNA-expressing vectors, we also investigated combinatorial vectors such as R4 (Gag5, Pol1, Pol47, and R/T5) and R3 (Pol1, Pol47, and R/T5). shRNAs that exhibit negative effects on cell growth such as Gag5 should be excluded from combinatorial RNAi vectors (Figure 6, Table 1). Removal of Gag5 from the R4 vector that exhibited impaired cell proliferation led to the design of the R3 lentiviral vector that scored no negative cell growth effects. Cytotoxicity by saturation of the cellular RNAi pathway is especially critical for the combinatorial shRNA vectors and might have contributed to the adverse R4 effects.

CYTOTOXICITY *IN VIVO*

Before one can proceed to a gene therapy trial in humans, safety should ideally be demonstrated in a preclinical animal model. As the RNAi mechanism is based on perfect sequence complementarities between siRNA and the viral target, the simian immunodeficiency virus/macaque model cannot be used for such studies. However, mice with the complete human immune system were created by injection of hHPCs into immunodeficient (BALB/c Rag2-/- IL2Rγc-/-) newborn mice. All major subsets of the human innate and adaptive immune system are found in the reconstituted HIS mice[22,23]. This HIS mouse model is ideally suited to test our gene therapy for several reasons. First, the hHPCs that are engrafted in the Rag-2-/-γc-/- newborn mice (Figure 7A) are similar to the ones that we propose to modify in our *ex vivo* gene therapy of





	GFP-				GFP+			
	T cells	4/8 ratio	B cells	Non T/B	T cells	4/8 ratio	B cells	Non T/B
JS1	26.5 ± 9.3	0.74 ± 0.23	60.2 ± 9.0	13.3 ± 2.1	21.9 ± 9.1	0.90 ± 0.26	56.2 ± 7.5	21.8 ± 4.1
shNef	20.8 ± 9.1	0.71 ± 0.11	67.8 ± 8.8	11.4 ± 2.2	18.2 ± 7.7	0.94 ± 0.21	55.1 ± 7.7	26.7 ± 5.0

Figure 7 ***In vivo*** **safety studies in the HIS mouse model.** A: Cell suspensions enriched for human hematopoietic progenitor cells (hHPC) are prepared from fetal liver tissue. Live nucleated CD34+ cells are magnetically sorted and further enriched for the hHPC (CD34+CD38- fraction) using fluorescence activated cell sorting. Lentiviral supernatants are produced on 293T cells. hHPC are transduced *ex vivo* with the shRNA-expressing lentiviral vector and injected intrahepatically into sub-lethally irradiated newborn BALB/c Rag-2-/-γc-/- mice. The transduction efficiency is evaluated based on GFP expression after 3.5 d in culture (GFP *in vitro*); B: The HIS (BALB-Rag/γ) mice are analyzed in the blood and the organs after at least 8 wk post-transplantation for the presence of human cells (%CD45+ cells) (left graph), which were analyzed for GFP recovery (right graph). The GFP recovery is the ratio between the frequency of human GFP+ cells measured in the animals (GFP *in vivo*) and the frequency of GFP+ hHPC injected in the newborn mice (GFP *in vitro*, transduction efficiency). The major subsets of the human immune system in the blood are also analyzed for their frequency and absolute number in the human GFP+ and GFP- population. Adapted from[24,70]. [a]$P < 0.05$.

HIV-1 seropositive patients. Second, as the hHPCs transplanted in the mice consist of a mixture of transduced (GFP+) and non-transduced hHPCs (GFP-), the human immune system in the animals will thus be constituted by transduced shRNA-expressing cells (GFP+) and non-transduced cells (GFP-). This provides an internal con-

trol to test for adverse effects of shRNA expression on hematopoiesis. Finally, as the HIS mouse can be infected by HIV-1, both the safety and efficacy of the shRNA therapy can be evaluated in the HIS mouse model.

The safety of an shRNA is assessed in the blood and the organs of the animals by multiple factors: (1) The presence of the human hematopoiesis-derived CD45+ GFP+ and GFP- cells; (2) the ratio between the frequency of human GFP+ cells measured in the animals and the frequency of human GFP+CD34+ cells injected in the newborn mice; and (3) the frequency and absolute number of different cell subsets of the human immune system, such as CD4+ and CD8+ T cells, B cells, monocytes and dentritic cells (Figure 7B). We previously tested the feasibility of an shRNA-based gene therapy in HIS mice reconstituted with hHPC transduced with a lentiviral vector expressing an shRNA against the HIV-1 nef gene[24]. In this model hHPC expressing anti-HIV shRNAs give rise to multi-lineage reconstitution of the human immune system *in vivo* and generate CD4+ T cells with the ability to resist HIV-1 replication in a sequence-specific manner. We tested our four candidate shRNAs and observed normal development of the human immune system in the animals for three of these shRNAs (Centlivre *et al* manuscript in preparation). A negative impact of Gag5 on the hematopoiesis of the HIS mouse was scored, confirming the *in vitro* findings in the CCG assay. These combined results led to the exclusion of Gag5 from the combination gene therapy (Table 1). The three "safe" shRNAs will now be combined into a single lentiviral vector for further *in vivo* safety tests.

PRE-EXISTING DRUG RESISTANCE MUTATIONS

Our proposed anti-HIV gene therapy will be developed for therapy-experienced HIV-1 infected individuals who have failed on regular antiretroviral drug regimens. As drug resistance mutations may affect the viral genome sequences targeted by RNAi, we investigated whether the target sequences of the top shRNA candidates are likely to acquire drug-resistance causing mutations. For this, we screened the Stanford HIV-1 drug resistance database[51,52]. The relevant drug resistance substitutions in the inhibited viral proteins are plotted in Table 1. In particular, the Protease gene sequence targeted by the set of overlapping shRNAs (Pol1-2, Pro1-7) has been implicated in the acquisition of resistance against Protease inhibitors like Nelfinavir, Aprenavir, Ritonavir and Indinavir at codons 30, 32 and 33. A treatment history that includes one of these Protease inhibitors and genotyping results that demonstrate the presence of at least one of these mutations will be an exclusion criterion for gene therapy participants.

COMBINATORIAL RNAi

The stable expression of anti-HIV shRNAs in T cells results in potent virus inhibition[16,53,54]. However, the application of a single shRNA inhibitor is not sufficient to maintain inhibition. Virus escape variants can emerge after extensive culturing[8-11,13,34]. Therefore, multiple antiviral shRNAs should be expressed simultaneously to achieve durable inhibition by raising the genetic threshold for viral escape[48,55,56]. This combinatorial strategy is analogous to current antiretroviral therapy regimens with multiple drugs that have led to significant clinical success in HIV-1 infected patients[57].

There are several ways to express multiple RNAi inhibitors against HIV-1, ranging from polycistronic miRNAs to extended multimeric shRNA transcripts[17-21]. We achieved most promising results with multiple shRNA cassettes[14,16]. To express multiple shRNAs, we initially inserted several H1-driven shRNA expression cassettes into the lentiviral vector. However, these vectors are extremely unstable as shRNA cassettes were deleted during transduction due to slippage of the Reverse Transcriptase enzyme on the repeated H1 promoter sequences[16,58]. To prevent recombination-mediated deletion of shRNA cassettes, we designed shRNA cassettes with unique promoter elements. The RNA polymerase III promoters H1, U6 and 7SK and the RNA polymerase II promoter U1 were used[4,59-61]. All these promoters have precise transcription start and termination sites and the shRNA expression levels are similar. The combination of four promoter-shRNA cassettes in R4 (U1-R/T5, U6-Pol1, 7SK-Gag5, and H1-Pol47) leads to durable virus inhibition in stably transduced T cells[16]. At present, the R4 combinatorial RNAi vector has been modified to R3 without Gag5 due to adverse cellular effects of this shRNA. The R3 lentiviral vector that confers the same potent and durable inhibition is proposed for the future clinical anti-HIV gene therapy trial.

CONCLUSION

We describe here the course that was taken to select the most potent and safe shRNA inhibitors against HIV-1, which will contribute to the development of an exclusively shRNA-based gene therapy against HIV-1. Currently, the combinatorial RNAi approach comprises three shRNAs targeting three distinct and highly conserved regions of the HIV-1 RNA genome.

The proposed RNAi gene therapy against HIV-1 will be developed for therapy-experienced HIV-1 infected individuals. During antiretroviral therapy, mutations can be selected in the genes that encode the drug-targeted viral proteins. Such mutations can interfere with RNAi attack when siRNA target sites are altered. Indeed, one shRNA inhibitor of the combinatorial shRNA vector targets a viral sequence that frequently acquires mutations to escape from Protease inhibitors. Patients who failed on Protease inhibitor containing regimens or that harbor viruses with such resistance mutations have to be excluded from the current combinatorial RNAi gene therapy. To overcome this issue, alternative shRNA regimens could be established that do not target viral genome regions

known to acquire prominent drug resistance mutations. Alternatively, one could attack the resistant HIV-1 strains with modified shRNA inhibitors. We previously showed that a combination shRNA strategy directed against the wildtype and drug-escape variants was able to efficiently and durably suppress virus replication[35,48].

The selection of HIV-1 escape variants must be prevented to durably suppress the chronic virus infection. To achieve this, targeting of conserved HIV-1 RNA regions is important, as well as the simultaneous application of multiple shRNAs. Potent virus inhibition will reduce the chance of virus escape by limiting the occurrence of mutations and the genetic threshold for resistance development is increased when multiple viral sequences are targeted. We continuously work on improvement of the shRNA design and recently identified loop sequences such as miRNA-derived loops that improve the siRNA processing and yield more potent gene knockdown and HIV-1 inhibition[62]. Alternatively, targeting of cellular cofactors that are essential for HIV-1 replication represents a promising anti-escape approach. 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. Recent screens revealed several cofactor-encoding mRNAs whose knock-down resulted in diminished HIV-1 replication[63-67]. However, knockdown of cellular proteins at the mRNA level might have negative effects on cell viability and anti-host shRNAs must be carefully designed. An ideal candidate cofactor is the CCR5 co-receptor. A natural deletion in the CCR5 gene (CCR5-Δ32) has been found at 1% frequency in the Caucasian population. These individuals are resistant to HIV-1 infection and do not appear to suffer from major biological effects or health issues due to the absence of this receptor protein[68].

Within a couple of years, RNAi has moved from the laboratory to clinical trials as novel therapeutic against a variety of diseases. In 2008, the first antiviral shRNA was used in combination with a TAR decoy and CCR5-ribozyme as an RNA-based gene therapy for HIV-1 infected individuals. The transfused cells were successfully engrafted and the anti-tat/rev siRNA was detected in peripheral blood mononuclear cells (PBMCs) up to 24 mo[69]. This initial clinical result provides encouragement for the anti-HIV gene therapy that we develop based exclusively on multiple shRNAs. The extensive preclinical assays in the humanized mouse model demonstrated the safety and efficacy of this combinatorial RNAi approach, which will soon move towards clinical testing.

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