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RNA interference-based therapeutics for human immunodeficiency virus HIV-1 treatment: synthetic siRNA or vector-based shRNA?

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Abstract

Importance of the field—Despite the extraordinary clinical benefits of HAART, the prospect of life-long antiretroviral regimen poses significant practical problems, which has spurred an interest in developing new drugs and strategies to treat HIV infection and to eliminate persistent viral reservoirs. RNAi is a highly potent natural gene silencing mechanism that has emerged as a novel therapeutic possibility for HIV.

Areas covered in this review—Our aim is to discuss the recent progress in overcoming the hurdles for translating transient and stable RNAi enabling technologies towards clinical applications in HIV infection and the review covers literature from the past 2–3 years.

What the reader will gain—HIV inhibition can be achieved by transfection of chemically or enzymatically synthesized siRNAs or by DNA-based vector systems to express short hairpin RNAs (shRNAs) that are processed intracellularly into siRNA. This review compares the merits and shortcomings of the two approaches, focusing on technical and safety issues that will guide the choice of the appropriate strategy for clinical use.

Take home message—Introduction of synthetic siRNA into cells or its stable endogenous production using vector-driven shRNA have both been shown to effectively suppress HIV replication in vitro and in some instances in vivo. Each method has its own advantages and limitations in terms of ease of delivery, duration of silencing, emergence of escape mutants and potential toxicity. Thus, both methods appear to have potential as future therapeutics for HIV, once the technical and safety issues unique to each of the approaches are overcome.

Keywords

antiviral therapy; HIV-1; RNA interference; short hairpin RNA; small interfering RNA

1. Introduction

Highly active antiretroviral therapy (HAART), the current standard treatment for HIV-1, has dramatically improved the prognosis of HIV-1 infected individuals.^{1–3} However, alternate therapeutic strategies are being developed due to the high cost, toxicity, patient non-compliance, and resistance associated with life-long HAART regimen.^{1, 2, 4–8} Particularly interesting in this regard is the emergence of RNA interference (RNAi) as a potential anti-viral therapeutic. The potency, specificity, and versatility of RNAi-mediated post-transcriptional gene silencing for suppression of acute as well as chronic viral infections

including influenza, SARS, flaviviruses, HIV, hepatitis C virus (HCV) and hepatitis B virus (HBV) has been clearly demonstrated in model systems.^{9, 10}

RNA interference (RNAi) is a phenomenon where small double-stranded RNA (dsRNAs) regulates specific gene expression. The biology and mechanism of RNAi have been extensively reviewed.^{11–13} Essentially, RNAi can be induced either by endogenously encoded small RNAs called microRNAs (miRNAs) or exogenously introduced small interfering RNAs (siRNAs).

In either case, the 21–23 nucleotide dsRNA associates in the cytoplasm with a protein complex called the RNA induced silencing complex (RISC), whereupon one of the two RNA strands (passenger strand) is degraded and the other “guide” strand guides the RISC to mediate sequence-specific degradation of the corresponding mRNA (in case of siRNAs and shRNAs) and/or translational repression by binding to the 3' untranslated region (UTR) (in the case of miRNAs). The main purpose of RNAi machinery in mammalian cells appears to be to generate small non-coding regulatory miRNAs. However, the existence of RNAi machinery also makes it possible for exotic designer small RNAs [synthetic siRNA or small hairpin RNA (shRNA)] to be used for silencing virtually any gene of interest in a sequence-specific manner. Ever since externally introduced double-stranded siRNAs were shown to silence specific gene expression in mammalian cells, there has been a tremendous interest applying them as potential novel drugs for the treatment of diseases.¹³

For specific gene silencing, RNAi can be induced by the introduction of chemically or enzymatically synthesized double-stranded siRNA or by intracellular generation of siRNA from vector driven precursor small hairpin (sh) RNAs. In the latter method, an oligonucleotide containing the siRNA sequence followed by a ~9 nt loop and a reverse complement of the siRNA sequence is cloned in plasmid or viral vectors to endogenously express shRNA which is exported out of the nucleus by exportin 5¹⁴, and is subsequently processed in the cytoplasm by Dicer into siRNA¹⁵ in association with dsRNA binding proteins like TRBP and PACT¹⁶. Due to the ease of delivery, particularly in primary cells, non-replicating, recombinant viral vectors (such as adeno, retro and lentiviral vectors) are commonly used for shRNA expression (Fig. 1). Because shRNA is continually produced within the cell, the gene silencing is long lasting (weeks to months). In contrast, synthetic siRNA effects are short lived (generally ~3–5 days) because of dilution with cell division and intracellular degradation. Also, synthetic siRNAs are not generally taken up by cells because of their relatively large size and net negative charge and thus, introduction of siRNA into cells requires the use of some form of delivery reagent (Fig.1).

RNAi technologies utilizing shRNA and siRNA have distinct advantages and limitations and thus, both strategies have been used to suppress HIV infection. This review will discuss the progress and challenges with both approaches, highlighting intrinsic differences between siRNA and shRNA with respect to delivery, duration of silencing and induction of off-target effects.

2. Viral versus host gene targets

RNAi can be used to silence several viral genes involved in the HIV-1 life cycle, starting with the incoming viral genome to the viral transcripts generated from the integrated provirus. Alternatively, cellular genes that impact multiple stages of the viral life cycle can also serve as potential therapeutic targets because HIV-1 heavily depends on the host cell machinery for entry, replication, egress and even persistence in a latent state.

2.1 Viral targets for RNAi

Several HIV sequences have been targeted by RNAi including the RNA sequences for structural *gag* and *env* proteins,^{17–19} the Pol enzymes,¹⁸ the infectivity factors *vif* and *nef*,^{6, 20, 21} the regulatory proteins *tat* and *rev*.^{6, 19, 22–25} as well as the non-translated RNA sequences in the long terminal repeat (LTR) domain.^{19, 20} An important unresolved issue is whether the presence of siRNA/shRNA can cure the cell of virus by destroying the viral genome before it is reverse-transcribed and integrated into the host genome. Although some studies reported that the amount of integrated provirus was reduced in cells pretreated with siRNA, the preponderant data suggest only a modest reduction in the level of integrated proviral DNA.^{23, 25–28} It remains possible that different siRNAs induce different effects but in general, de novo made viral transcripts rather than the incoming genomic RNA appear to be susceptible to RNAi-mediated degradation.

The propensity of HIV-1 to mutate its sequence poses a serious challenge for designing effective antiviral therapeutics based on an exquisitely sequence-specific strategy like RNAi.^{29–32} HIV-1 can escape siRNA/shRNA-mediated control through single or multiple nucleotide mutations or even by the deletion of the whole region containing the siRNA recognition site.³⁰ Moreover, there is evidence that HIV-1 can undergo nucleotide substitutions that induce alternative RNA folding, thus shielding a previously targeted sequence from being accessed by the siRNA.³¹

One strategy to thwart the ability of the virus to evade restriction by RNAi is to target viral sequences that are highly conserved across the various clades and strains.⁸ RNAi target sequences have been identified in the HIV-1 genomic regions that have essential roles in maintaining structural and functional integrity of the virus.^{8, 33–35} In fact, a large number of relatively conserved HIV-1 sequences have been identified for potential RNAi targeting.^{36, 37} In one study, a highly conserved *vif* target sequence was able to protect CD4 T cells from all the HIV-1 clades, including multiple isolates of clade B, prevalent in the West.⁸ An even more robust strategy is to simultaneously target multiple discrete and relatively well-conserved sequences. The likelihood that a single viral RNA molecule would mutate all targeted sequences becomes increasingly small as the number of targets is increased.³⁶

2.2 Host gene targets for RNAi

The difficulty in addressing the high rates of viral mutation has led to an emphasis on using cellular targets as candidates for HIV therapeutics with RNAi. With increasing knowledge of HIV biology, targeting relevant cellular genes to clear both circulating and latent viral reservoirs may become possible, which could prove more effective than mere suppression of actively replicating virus by silencing viral genes. RNAi targets that facilitate viral entry are ideal for inducing HIV resistance in susceptible cells as the virus would be unable to initiate the infection.¹⁷ Many studies have targeted the viral receptor CD4^{17, 34, 38}, and the co-receptors CCR5^{6, 7, 38–40} and CXCR4^{38, 41, 42} that are essential for attachment of the HIV-1 particle to the cell and subsequent viral entry. CCR5, a co-receptor for the macrophage-tropic virus that predominates during early infection, holds particular promise as a therapeutic target because a 32-base pair deletion of the gene is known to be relatively well tolerated and at the same time confer resistance to HIV infection.⁴³ The validity of CCR5 as a therapeutic target has received further boost from a recent case study where a HIV seropositive patient transplanted with stem cells from CCR5delta32 homozygous donor remained virus-free without HAART drugs up to 20 months after transplantation.⁴⁴ Even individuals who are heterozygous for this deletion have delayed progression to AIDS.^{45–47} However, a potential problem is the ability of HIV-1 to switch tropism to CXCR4 during the course of AIDS, which could create a more virulent infection.⁴⁸ To circumvent this

possibility, dual-specific shRNAs that combine anti-CCR5 and CXCR4 shRNAs have been engineered and down-regulation of these receptors has been shown to virtually shutdown viral infectivity in human lymphocytes.^{38, 49, 50} However, a cautionary note on targeting the T cell tropic co-receptor CXCR4 is that the molecule may be required for hematopoietic stem cell (HSC) homing to marrow and subsequent T cell differentiation.^{51–53}

Many other host factors important for HIV-1 replication have also been successfully targeted for RNAi, including the transcription factor NF- κ B, cyclin T1, cyclin-dependent kinase 9, or suppressor of Ty5 homolog, but they may be of limited therapeutic utility because of their important roles in cellular physiology.^{54–56} With increasing knowledge of HIV-host cell interaction through recent advances in high throughput genomic and proteomic screens and bioinformatics prediction, the repertoire of potential host RNAi targets for inhibiting HIV replication is rapidly expanding. Three independent studies have used the power of RNAi itself for identifying novel host cell genes linked to HIV replication.^{57–59} Although the results from these and multiple other genome wide screens have been highly divergent with respect to the host genes identified (possibly because of differences in the target cells used for infection and the varying sampling times), nevertheless the data sets do show overlap for some of the identified genes.^{57–59} Particularly interesting from a therapeutic standpoint are the retrograde golgi transport proteins Rab-6 and Vps53. siRNA knockdown of either of these proteins was sufficient to block HIV replication at a very early step, even before reverse transcription of the viral genome, with no deleterious effect on the cell viability.⁵⁸ Importantly, Rab-6 knockdown efficiently suppressed both R5 and R4 tropic viruses, suggesting that it could provide a better target than the co-receptor CCR5. Similarly, knockdown of the cellular transportin 3 (TNPO3) blocked HIV at the next stage after reverse transcription but before integration of the viral genome.⁵⁸ If these and other putative HIV-dependency factors can be verified as relevant for infection of primary cells and their ablation is well tolerated, they would provide a major cache of potential therapeutic RNAi targets for treating HIV infection.

A novel possibility for intervention has recently emerged based on the knowledge about the role of cellular miRNA machinery in maintaining HIV in a productive or latent state. Certain cellular miRNAs, in particular miR-28, miR-125b, miR-150, miR-223 and miR-382 have been reported to significantly contribute to the maintenance of viral latency.⁴⁶ In fact, neutralization of these miRNAs using antagomirs was enough to induce virus production from latently infected CD4 T cells, opening up the possibility of manipulating select cellular miRNAs for reversing viral persistence.

Given the special challenges posed by the HIV life cycle and genetic variability, use of a combination of siRNAs/shRNAs targeting conserved viral sequences and nonessential host genes important in the viral life cycle may be the optimal strategy to inhibit HIV at several stages of its life cycle. For HIV gene therapy, the emerging consensus is to combine RNAi with other HIV inhibitory approaches such as ribozymes, RNA decoys, transdominant proteins, species-specific restriction factors like TRIM5 α , antisense RNA and aptamers to further safeguard against viral escape akin to antiretroviral drug cocktails.^{3, 60–62} In fact, a clinical trial is underway with a triple combination lentiviral construct comprised of a TAR RNA decoy, shRNAs targeted to *tat* and *rev* open reading frames and a chimeric anti-CCR5 *trans*-cleaving hammerhead ribozyme.³

3. Synthetic siRNA for HIV inhibition

Advances in understanding the RNAi pathway has led to substantial improvements in rational design of siRNAs for potential use as a drug for treating diseases. From an understanding of the thermodynamic features of siRNA loading in RISC, it has become

clear that the duplex should be designed so that the antisense guide strand is less stable at its 5' end thereby favoring its uptake by RISC. Another novel design feature is the use of longer duplex RNA with a 2 nt 3' overhang only at the antisense end in place of the short 19–21 nt siRNA.⁶³ The longer form has been demonstrated to trigger more potent gene silencing because it acts as a dicer substrate and the dicer cleaved product allows more efficient RISC loading.^{64, 65}

Despite the rapid advances and great promise, developing siRNA as an antiviral drug for HIV-1 remains a challenge. Delivery is a major hurdle as mammalian cells and tissues do not spontaneously take up negatively charged molecules like siRNA.^{66–68} The susceptibility of siRNA to endogenous nuclease is also an impediment but can be overcome by introducing chemical modifications in the siRNA or by binding to or encapsulation of siRNA within the delivery reagent. Thus, therapeutic success will hinge on developing practical delivery reagents that are capable of protecting siRNA in circulation as well as of delivering it to the cytoplasm of appropriate cell types *in vivo*.⁶

A number of non-viral carriers have been tested for potential *in vivo* delivery of siRNA including cationic polymers, peptides or liposomes and lipid-like materials that form complexes with negatively charged siRNAs by ionic interactions.^{69–70} The resulting complexes not only allow cellular uptake of siRNA via the endocytic pathway but also provide excellent protection from nuclease attack. For example cationic PEGylated liposomes called 'stable nucleic acid lipid particles' (SNALPs) have been used to deliver siRNA to the liver in cynomolgous monkeys to reduce serum cholesterol and LDL levels.⁷¹ A significant development is the use of siRNA carriers that display various ligands (e.g. antibodies, peptides, and sugar chains) that bind to specific cell surface proteins.^{6, 70} One study used a fusion protein of the Fab fragment of antibody against HIV gp160 with the positively charged protamine that enables siRNA binding.⁷² This reagent effectively delivered siRNA to HIV-infected cell lines and primary T cells *in vitro*. Moreover, systemic treatment in mice resulted in cell-specific targeting to HIV envelope-expressing melanoma cells. This initial demonstration of the feasibility of selective delivery to HIV-infected cells was followed by a more generally applicable technique of targeting specific cell-surface proteins of immune cells. A single chain antibody variable fragment (scFv) to the CD7 receptor conjugated to a nucleic acid binding nonamer arginine peptide (9R) was successfully used for T cell-specific delivery of antiviral siRNA.⁶ In this study, it was possible to suppress HIV-1 replication and prevent CD4⁺ T cell depletion *in vivo* in humanized NOD/SCIDIL2ry^{-/-} mice {reconstituted with human lymphocytes (Hu-PBL) or CD34⁺ hematopoietic stem cells (Hu-HSC)} by simple intravenous injections of a combination of siRNAs targeting the viral *vif* and *tat* genes along with the host CCR5 gene. Importantly, CD7 mediated delivery of siRNA successfully silenced the target gene in naive/resting T cells⁶ that are vulnerable to infection when they become activated and also serve as latent reservoir that can rekindle viral replication after interruption of HAART. The CD7-specific antibody is well suited for siRNA delivery because CD7 is expressed by most T cells and is rapidly internalized after binding of the antibody-siRNA complexes.⁶ Moreover, this antibody has already been used in clinical studies to target toxins to T cell lymphomas and leukemias.⁶⁶ Another antibody directed to a predominant integrin on human leukocytes, LFA-1 was also shown to deliver siRNA to immune cells when expressed as an antibody-protamine fusion protein.⁷³ Although the antiviral efficacy *in vivo* remains to be tested, an advantage over CD7 scFv, which selectively targets T cells is that the LFA-1 antibody would permit siRNA delivery to a broader spectrum of HIV susceptible cell types, including T cells, macrophages and dendritic cells that play key roles in infection and pathogenesis.⁷³ In fact, in a recent study, a nanoparticle formulation coated with this LFA-1 antibody (LFA-1 tsNPs) was able to efficiently deliver anti-HIV siRNAs to T cells and macrophages *in vivo* and reduce plasma viral load and CD4 T cell loss in the humanized mouse model.⁷⁴

For any antibody-based delivery reagent, the prospect of repeated *in vivo* administration brings in the risk of triggering immune response to the antibody itself. For instance, in a nonhuman primate model, reinjection of human transferrin conjugated polymers elicited antibodies to transferrin.⁷⁵ Perhaps fully or partially optimized humanized antibodies can be used to reduce potential immunogenicity of targeting moieties.⁷⁶ The antibody amounts could also be reduced by use of delivery vehicles like tsNPs for in this case, unlike methods where siRNA binding is through positively charged residues linked to the antibody, the antibody only serves as a targeting ligand and thus large payloads of siRNA can be packaged without the need to increase the antibody levels on the surface of the nanoparticle.

A novel non antibody-based delivery vehicle in which the protein transducing domain (PTD) from the HIV TAT protein is attached to a double-stranded RNA-binding domain (DRBD) that binds siRNA with high avidity was also shown to efficiently deliver siRNA to T cells *in vivo*.⁷⁷ However, the PTD-DRBD-delivered system is not selective to T cells as it can induce RNAi in many different primary and transformed cell types. This could be a disadvantage in that targeted delivery restricted to the relevant cell types improves the therapeutic availability of siRNA *in vivo* thereby reducing the siRNA dose needed. This might also be important to minimize the risk of undesired potential of off-target effects in non-targeted bystander cells.^{66, 78}

4. shRNA for HIV inhibition

On a parallel track with the development siRNA-based technologies for HIV inhibition, shRNA-expressing DNA vectors have also been developed for potential long-term suppression of the virus. Viral vectors like adenovirus (AdV), adeno-associated virus (AAV), and retroviruses like oncoretrovirus and lentivirus (LV) are generally used for shRNA expression (reviewed in ^{79, 80}). Lentiviruses are particularly suited for gene therapy because they integrate into the host genome to provide life-long expression of the shRNA transgene. Another advantage of lentiviruses is that they can deliver large genetic payloads, a property that can be exploited for expressing multiple shRNAs simultaneously.³⁶ Although oncoretroviruses are also capable of integration into the host genome, they depend upon nuclear membrane breakdown during cell division to transduce cells, whereas lentiviruses can transduce quiescent cells as they use their own active nuclear import pathway for integration.^{81, 82} Lentiviruses, like oncoretroviruses (e.g., MLV based vectors), have been rendered progressively safer by the development of split plasmid systems for vector production to prevent generation of replication competent virus (reviewed in ^{80, 83}). The basic idea here is to generate a virus that after a single round of infection ensures stable integration of the transgene with only a minimal component of the viral genome, so that the transgene is continually expressed but infectious virus is not generated.⁸⁴ This system also avoids HIV envelope and instead uses a substitute envelope for pseudotyping lentiviral particles, which also serves to broaden the tropism of the vector. Most commonly used for this purpose is the vesicular stomatitis virus glycoprotein (VSV-G), which is resistant to ultracentrifugation and freeze-thaw cycles and that can enter the host cell via the endocytic pathway thereby reducing the requirements of HIV accessory proteins for infectivity.⁸⁵

Lentiviral pseudotyping methods that allow targeted delivery to HIV susceptible cells are also being developed to improve transduction efficiency and to facilitate direct administration into the bloodstream. One approach has used lentiviral vectors pseudotyped with retroviral envelope proteins fused to targeting molecules, such as single-chain antibodies or cytokines.^{86, 87} In another approach, the Fc-binding region of protein A (ZZ domain) has been inserted into the original receptor-binding region of the Sindbis virus envelope protein used for lentiviral pseudotyping.⁸⁸ The zz domain serves as a versatile adapter molecule as any cell targeting antibody can be bound through the interaction with

the Fc region of antibodies. This approach has been applied for generating a lentiviral vector tagged with CCR5-directed monoclonal antibody. This reagent was shown to transduce a CCR5 shRNA into HIV-susceptible cells and confer resistance to HIV-1 infection.⁸⁹ More recently, a biotin adaptor peptide has also been similarly inserted into the same site in the Sindbis envelope protein to permit high affinity binding of the biotinylated vector to avidin-antibody fusion proteins, which may provide a more stable reagent for *in vivo* targeting.⁹⁰

The possibility of targeting its own sequence which is always a part of the vector genome is a theoretical disadvantage with shRNA expressed from a lentiviral vector but studies show that such self targeting does not actually occur.⁹¹ On the other hand, shRNAs sequences that target the viral Gag-Pol region can affect the generation of the transducing lentivirus by targeting the Gag-Pol genes that are provided *in trans* for packaging the lentiviral vector. However, the problem can be circumvented with the use of human codon-optimized gag-pol gene in the packaging construct to avoid reducing transducing viral titers while retaining gene silencing activity in the context of HIV-1 infection.⁹²

4.1. shRNA design

In its earliest form, vectors for endogenous siRNA generation were engineered to express templates for precursor short hairpin RNA (shRNA) that consist of a sequence of 21–29 nt, a short loop region, and the reverse complement of the sequence and a short terminator (5–6 T residues) sequence. Because the expressed RNA is short, generally Pol III promoters such as U6 or H1 were used (Figure. 2). These promoters are strong and generate large amounts of transcription products (although H1 promoter is weaker than U6) that serve as substrates for Dicer. On the downside, over expression of U6 driven shRNA could be toxic presumably due to interference with cellular miRNA expression.^{93, 94}

Recently miRNA-based lentiviral vectors have been used for expressing shRNAs. shRNA embedded in microRNA scaffold provide more robust expression of siRNAs and gene silencing as compared to conventional shRNA constructs.⁹⁵ Pol II driven polycistronic transcripts containing multiple shRNA sequences can be efficiently expressed from the miRNA backbone. This is a major advantage in treatment of HIV infection as expression of a single sh/siRNA can lead to the emergence of escape mutants.³¹ In two recent studies simultaneous expression of multiple anti-viral shRNAs from the six pre-miRNA-encoding mir-17–92 cluster or a polycistronic miR-106b cluster (miR-106b, miR-93, miR-25) had better anti-HIV efficacy than individual shRNAs.^{96, 97} miR-30 and mir-155-based artificial vectors have also been used for expression of antiviral shRNAs. In one study expression of anti-HIV shRNA from a mir-155-based vector demonstrated robust and sustained gene expression for over four weeks.⁹⁸ These features are a big plus for *in vivo* application of mir-based shRNA vectors. Another positive conclusion from these studies is that template sequences inserted in a miRNA backbone are less likely to compete with the endogenous miRNAs for transport and incorporation into the RISC complex. Moreover, unlike conventional shRNA constructs that rely on the integration of multiple copies in the host genome, miR-based shRNA can knockdown gene expression efficiently even at a single copy integration.⁹⁹ Induction of innate antiviral responses is also reduced with the use of shRNAmiRs, probably because with assimilation into the miRNA pathway the shRNA generation occurs by the natural Drosha and Dicer processing.¹⁰⁰

4.2 Conditional shRNA vectors

Inducible expression of transgenes provides an improved level of safety as it avoids much of the unintended consequences of viral vector-mediated delivery and gene silencing. Reversible conditional vectors are usually drug-inducible (e.g. tetracycline) and can be expressed from a pol III or pol II promoters. Repression can be achieved by steric hindrance,

as with tetracycline binding to the Tet repressor (tetR) thereby sequestering off the Tet operator (tetO), or by transactivation which relies on the expression of an engineered pol III transactivator (tTA or rtTA), which in turn induces transcription of shRNA from a modified U6 promoter.¹⁰¹ In another elegant approach, anti-HIV rev shRNA was cloned under a pol II hsp70 promoter that could be transactivated by HIV-1 TAT, downstream of an LTR sequence.¹⁰² In the absence of TAT, transcription from the LTR was stalled at the TAR sequence in the LTR and no shRNA was made. However upon TAT induction, it was able to bind the TAR element and relieve the transcriptional block by recruiting RNA polymerase to initiate shRNA expression.¹⁰² The main advantage of this system is that the shRNA expression is only induced in HIV-1 infected cells, thus avoiding non-specific toxic effects.

Proof of concept studies in humanized immunodeficient mouse models have shown that CD34+ HSC's transduced with lentiviral shRNA can differentiate into monocytes and/or T cells.^{103–105} However, in these studies HIV inhibition has only been validated ex vivo using T cells and macrophages differentiated within xenografts transplanted with human CD34+ HSC. In a study that augurs well for human gene therapy for HIV, the Chen group has demonstrated that the strategy also works in a rhesus macaque hematopoietic stem/progenitor cell transplant model that more closely mimics humans.¹⁰⁶ In two animals reconstituted with CD34+ hematopoietic stem cells transduced with H1 promoter-driven shRNA targeting CCR5, about 5% of progeny lymphocytes were shown to express the transgene for up to 14 months. Another encouraging feature was the lack of vector or promoter related toxicity during this period of observation despite the use of a potent shRNA that consistently downmodulated gene expression. However, as in the mouse model, resistance to simian immunodeficiency virus infection was only demonstrated ex vivo. Thus, realizing the full promise of RNAi gene therapy may require further refinements to achieve gene marking levels that permit direct virus challenge in vivo. More rigorous studies in preclinical models would also be required to validate the approaches and satisfactorily allay the concerns of vector toxicity, insertional mutagenesis, as well as the possibility of emergence of replication competent viruses.

5. Toxicity and bio-safety

Better understanding of mechanisms that lead to toxicity and bio-safety issues are essential before RNAi can become a practical therapeutic. Important aspects related to toxicity that needs consideration include immune activation and off-target effects.

Immunostimulation by RNA-based gene therapy is a potential concern as intracellular presence of dsRNA can activate components of the innate arm of the immune system like cytosolic dsRNA-activated protein kinase PKR and retinoic acid-inducible gene (RIG-I) systems that lead to a type I interferon (IFN) response and further activation of IFN-regulated genes.^{107, 108} For exogenously introduced siRNAs, the induction of innate immunity is dependent on the siRNA structure and sequence, method of delivery, and cell type. In particular, immuno-stimulatory sequences that contain 5'-UGUGU-3' or 5'-GUCCUCAA-3' have been shown to interact with different endosomal Toll-like receptors (TLRs) leading to signaling cascade that elicits an IFN α production in dendritic cells¹⁰⁹. In some cases the delivery vehicles used for inducing siRNA uptake by cells can themselves induce an inflammatory response.¹¹⁰ Similarly, shRNA, expressed from the pol III promoters generally have a triphosphate at their 5' ends that can activate RIG-I to induce an interferon response, resulting in the activation of PKR and subsequent shut down of cellular translation.^{107, 108}

Both siRNA and shRNA can induce off-target effects by fortuitous base pairing with unintended mRNAs resulting in suppression of important cellular components.^{111–113}

Complementarity with 2–7 nucleotides at the 5' end of the guide or passenger strand has been shown to be a key determinant in directing off-target effects.¹¹¹ Off-target effects can be reduced by avoiding the incorporation of sense strand of siRNA into the RISC complex. Chemical modifications that include 5' methyl or 2'-O-methyl ribosyl substitution at position 2 in the guide strand can effectively avoid sense strand-RISC formation and reduce off-target silencing.^{114–117} Also incorporation of two nucleotide overhangs at the 3' end of only the antisense strand can reduce off-target sequences by favoring its preferential incorporation to RISC.¹¹⁸ Additionally, use of software such as Smith Waterman and dsCheck,^{119, 120} for alignment analysis of short sequences may further avoid induction of off-target miRNA-like effects by the selected siRNA sequences. The advantage with synthetic siRNA over shRNA is that it not only permits specific chemical modifications to reduce off-target effects but also provides the flexibility to quickly modify the sequence if it negatively affects the potency of gene suppression.

Since the RNAi pathway shares the cellular endogenous miRNA machinery, with both siRNA and shRNA, there is an opportunity for competition with miRNAs for loading to the RISC.¹²¹ Because miRNAs are essential for the regulation of mammalian cell growth, differentiation, and apoptosis, if the machinery for miRNA biogenesis is diverted to handle exogenous siRNA or shRNA, then a dearth of miRNAs can potentially manifest as toxicity.^{122, 123} In mice, severe liver toxicity and fatality were reported within 2 weeks of shRNA treatment which could be attributed to the saturation of the miRNA pathway by over-expressed shRNA.¹²⁴ The problem may be more easily overcome with synthetic siRNA where the dose administered can be controlled. In the case of vector encoded shRNA, the problem will have to be overcome by the use of weaker promoters to generate potent shRNAs that work at low concentrations or by expressing shRNA from miR-based vectors that assimilate more seamlessly into the endogenous pathway. In a preclinical study in the rhesus macaque model, a potent shRNA sequence directed at CCR5 was found to be highly effective and nontoxic when expressed from the H1 Pol III promoter in place of the much stronger U6 promoter.¹⁰⁴

For clinical use of lentiviral vector for shRNA expression, insertional mutagenesis is a potential hazard to be considered. In this respect, lentiviral vectors are considered much more safer than gammaretroviral vectors as they have less predilection for integration into transcriptionally active sites. However, in a recent study that compared the transformation capability of the two vectors in a sensitive assay system, a SIN lentiviral vector was also shown to be capable of triggering transformation of primary hematopoietic cells although at a much lower frequency than a SIN gammaretroviral vector. A common insertion site (CIS) in the first intron of the *Evi1* proto-oncogene could be identified in the lentiviral vector-transformed mutants¹²⁵. On a more optimistic note, the study also noted that the mutagenic capability of the vector could be reversed by altering its enhancer–promoter elements.

Conclusions and Perspective (Expert opinion)

Synthetic siRNA or vector-based shRNA?

In cultured cells, HIV inhibition has been effectively achieved with both siRNA or vector expressed shRNA approaches. However, tailoring RNAi-based intervention for a complex virus like HIV that is able to adapt so cleverly to drugs and immune pressure presents formidable challenges. Both the transient siRNA and stable shRNA approaches have their unique advantages and disadvantages with respect to duration of gene silencing, toxicity and delivery efficiency (Table. 1), which will have to be considered carefully for use of RNAi as therapy.

Long-term endogenous expression of siRNA from vector-derived shRNA is probably the most optimal strategy for a persistent infection like HIV. The major advantage being the possibility of achieving durable viral suppression without the repeated administrations required with exogenously introduced siRNA. Further, although synthetic siRNA can be loaded onto RISC for RNAi function, the loading process is generally less efficient than shRNA that is processed by the endogenous RNAi machinery.^{126,64}

As HIV pathogenesis is primarily driven by depletion of CD4⁺ T cells, one possible practical form of shRNA therapy is to infuse an in vitro expanded population of autologous T cells that are genetically modified to express antiviral shRNAs. In a recent study, infusion of CD4⁺ T cells transduced with lentivirus expressing an antisense oligonucleotide against the HIV-1 envelope gene was tested in humans with no adverse effects and up to 4% of CD4⁺ cells expressing the transgene.¹²⁷ However, although there may be short-term benefit with this form of therapy, transfused T cells decline over time and repeated infusions could adversely affect the natural T cell repertoire. A more optimal gene therapy option would be to transduce hematopoietic stem cells so that the DNA precursor for the shRNA becomes a permanent part of their genome and their progeny cells. As the gene modified HSCs would be capable of self-renewal, theoretically they could serve as a life-long source of HIV resistant multiple cell lineages (including T cells, macrophages, dendritic cells). Lentiviral vectors are well suited for HIV gene therapy as they have the ability to efficiently transduce primary T cells and quiescent stem cells. However technical aspects of lentiviral gene therapy need more refinement. At the current stage of development, resting T cells are far less amenable to transduction than activated cells. Even HSCs can be transduced efficiently only after ex vivo culture in a cytokine cocktail that is likely to compromise pluripotency, leading to inefficient generation of gene marked progeny blood cells. Variable gene expression during in vitro differentiation can further compound the problem.¹²⁸ Strategies such as using insulators (like *chs4* element, sea urchin *Ars*) that resist DNA methylation or replacement of U3 region of the viral LTR with a DNase hypersensitive HS2 enhancer element region from the erythroid GATA-1 transcription factor gene are being tested to reduce epigenetic silencing without loss of viral titer, gene expression, and genomic stability in HSCs.^{129–131}

If the delivery hurdle can be overcome, despite the transient nature of gene silencing, synthetic siRNA as a drug offers several obvious advantages over vector-driven endogenous expression. The simplicity of design, manufacturing, and testing, combined with the ability to stop the drug in case of any toxic effects, make it potentially easier and safer to use than vector-based shRNA therapy. Additionally, studies in preclinical HIV models suggest that with the use of a suitable delivery vehicle, siRNA uptake can be induced in a large proportion of vulnerable cells.^{6, 70} It is doubtful if such level of gene marking can be achieved with a shRNA gene therapy approach. Another significant benefit of siRNA over shRNA is the short time required for target screening. In the context of HIV-1, the ability to quickly change siRNA sequences to keep pace with the mutating virus would be an important advantage. The inherent flexibility in changing siRNA combinations would also allow testing multiple combinations of siRNAs. In addition to viral gene targets, many host molecules that play an important role in HIV-1 life cycle⁵⁸ can be transiently perturbed to delay primary infection or curb viral dissemination or even reverse latency by use of antagonists to cellular microRNA that modulate HIV gene expression.¹³²

Synthetic siRNA also offers the exciting possibility of application as a microbicide for protection against HIV at the port of entry. Local vaginal delivery of synthetic siRNA has been demonstrated to protect mice from lethal herpes simplex virus challenge.^{133, 134} The level of difficulty is likely to be higher for using the strategy for HIV infection as unlike HSV-2, where resident epithelial cells have to be protected, in the case of HIV, delivery of

siRNA will have to be accomplished across the epithelial barrier into populations of immune cells that are resistant to nucleic acid uptake. Moreover, because of their constant influx and egress, hematopoietic cells that initiate HIV infection at the site may be more “hard to hit” targets.

Conclusions—Both shRNA and siRNA are strong contenders as potential treatment options for HIV infection. Taking into consideration the unique advantage that each of the platform has to offer, it may be wise to explore both strategies in parallel. With further advances, it may even become possible to combine the two approaches so that gene-modified CD34⁺ HSCs are used to ensure long term generation of HIV resistant cells whereas “siRNA drugs” are used in the interim or for transient silencing of additional host genes whose long-term ablation is poorly tolerated. So far the preclinical studies in mouse and rhesus macaques and more recently the clinical studies in humans have not yielded any unprecedented toxicity or side effects that preclude the use of siRNA delivery platforms or shRNA vectors for treating HIV. Results of the ongoing clinical trial with vector-based shRNA and others to come will determine whether RNAi-based therapeutics can become an active player in the treatment of HIV infection. In conclusion, the exciting advances reviewed here provide great optimism for future applications of both siRNA- and shRNA-based RNAi therapies for HIV.

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Highlights

- Viral and cellular gene targets
- Vehicles for delivery of anti-viral synthetic siRNAs
- Lentiviral therapy to generate HIV-resistant T cells and macrophages
- Generation of multiplexed anti-viral shRNA from miRNA-based vectors
- Toxicity and biosafety issues for si/shRNA
- Scope of siRNA and shRNA for anti-HIV therapy

Figure 1. Promising strategies for using si/shRNA for HIV infection

The most favored method for expressing shRNA is infection with self inactivating lentiviral particles (generated using transfection with a three-plasmid system (shRNA expression cassette, packaging construct and an envelope construct). After infection, the shRNA expression cassette integrates in the cell's genome leading to long-term production of shRNA that is processed in the cytoplasm into siRNA. The most promising targeted siRNA delivery strategy consists of a targeting and a cargo moiety. Examples include gp120 Fab-protamine, CD7ScFv-9R protein and siRNA-encapsulated immunoliposome coated with LFA-1 antibody. After delivery, the targeting complex is endocytosed and siRNA released from the endosome is taken up by RISC to mediate gene silencing.

Figure 2. si/shRNA design to enhance potency and reduce toxicity

Short and long siRNA triggers and chemical modifications of siRNA are depicted on the left. Strategies for conventional shRNA, shRNAmiRs and conditional shRNA expression are depicted on the right

Table 1

Synthetic siRNA versus vector-based shRNA for HIV therapy

	siRNA	shRNA
Duration of knockdown	Transient (days)	Stable (months to years)
Chemical modification	Can be altered to increase stability and specificity	Natural, limited
Cloning/construction	Not required	Required
Production throughput	Fast	Slow
Targeting multiple sequences	Easy	Possible but slow/expensive
Combination Therapy	Few options	Can be combined with other anti-HIV gene therapy approaches.
Delivery formulation	Special carriers required for in vivo delivery to hard to transfect primary T cells and macrophages	CD34+ HSCs or primary T cells have to be transduced ex vivo with gene therapy vectors.
In vivo gene marking	Good with appropriate delivery strategy	Poor but proportion of gene marked cells would increase due to selective advantage during infection
HIV inhibition in vivo	Good in humanized mice	Not tested in animal models. Clinical trial underway.
Toxicity	Off target effects, interferon induction and immune response to delivery vehicle	Off target effects, interferon induction and insertional mutagenesis
Generation of replication competent virus	Not applicable	Potential hazard
Long term safety	Controllable	Uncontrollable