REVIEW

# Therapeutic Potential of RNA Interference Against Cellular Targets of HIV Infection

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**Abstract** RNA interference is not only very promising in identifying new targets for drug development, siRNA/shRNA themselves may be directly used as therapeutic agents. In inhibiting viral infections by RNA interference, both viral targets and cellular proteins have been evaluated. Most of the early studies in this field had chosen viral targets for RNA interference. However, recent efforts are mainly focusing on cellular proteins for RNA silencing due to the realization that a variety of viral responses substantially minimize siRNA effects. With the application of siRNA approaching, many new cellular targets relevant to HIV infection have been identified. The value of siRNA/shRNA in the treatment of AIDS is largely dependent on better understanding of the biology of HIV replication. Efforts in the identification of cellular processes with the employment of siRNA/shRNA have shed some new lights on our understanding of how HIV infection occurs. Furthermore, the relative specific effects and simplicity of design makes siRNA/shRNA themselves to be favorable drug leads.

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## Introduction

In the past few years, RNA interference (RNAi) has become a powerful tool for associating genotype with phenotype [1]. Unlike classical reverse-genetics approaches such as the production of gene knockouts, RNAi-mediated cleavage of specific cellular RNAs permits loss-of-function experiments to be conducted for virtually any target on time-scales and at costs typically associated with standard cellular biology experiments. Increasingly, large libraries of chemically synthesized, double-stranded small-interfering RNAs (siRNAs) or transcribed short-hairpin RNAs (shRNAs) are employed in cellular models to interrogate relationships between each target in the library and the phenotypes in question, and such approaches are very likely to yield insights leading to the identification of novel therapeutic targets or modulators of key cellular processes [2]. RNAi has also been used extensively to confirm the activity of targeted gene products at the level of single genes and is now a commonly deployed research tool for precision biology.

While the utility of RNAi for target discovery has been well established, the potential to deplete in vivo targets also makes RNAi an attractive alternative to small molecule agonists or antagonists, particularly for proteins lacking small molecule binding activities or non-coding RNAs, where finding compounds capable of disrupting their activity in disease processes may prove extremely difficult [3–7]. An area of active use of RNAi both for target discovery and potential therapeutic application involves attempts to block key steps in viral infection or

pathogenesis, notably for RSV and HIV. Initial conventional therapeutic strategies focused on identifying viral factors that could be effectively targeted with small molecules [8, 9]. The low fidelity of viral replication has evidently resulted in rapid mutation of viral genome however, permitting viruses rapidly to develop drug-resistant mutants [10]. Therefore, increasing potential cellular target list beyond classically drugable targets through the use of RNAi-based therapeutics likely represents a new avenue for target selection.

With respect to the inhibition of viral infections by RNAi, both viral and host cell targets have been evaluated. While most early studies had chosen viral targets for RNA interference, recent efforts have increasingly focused on RNAi-mediated inhibition of host cellular proteins required for viral infection due to the realization that a variety of viral responses substantially minimize siRNA effects. With the application of siRNA approaching, many new cellular targets relevant to HIV infection have been identified (Table 1). These newly identified cellular proteins offer

great potential in the development of both siRNA and other types of drugs in the treatment of HIV infection. Several recent review papers provide comprehensive information regarding the potential of viral targets of siRNAs in inhibiting viral infections [9–12]. This article focuses on the therapeutic potential of RNAi against cellular targets of human immunodeficiency virus (HIV) infection, initially describing the disadvantages of viral targets and then introducing some of the cellular proteins that have been studied using siRNA approaches to combat viral infections.

## Therapeutic Potential: RNAi can Knockdown both Viral and Host Genes

The list of viruses targeted by RNA interference is rapidly expanding. In the past few years, all major types of viruses have been chosen for siRNA studies, including HIV, hepatitis B, C and D, SARS, influenza virus, poliovirus, Estein-Barr virus, Adenovirus Human herpesvirus 6, and

**Table 1** List of cellular targets of HIV infection evaluated by RNAi

| Mechanism | Cellular target | References (PMID#)[a] |
|---|---|---|
| HIV entry[b] | CCR5, CXCR4, CCR4, CCR7, CD4, D6, CD11c, CD44, CD47, CD68, CD74, CSF3R, GABBR1, TNFR2 | 14527694; 15000819; 14581533; 12518064; 12461411; 15306840; 15051386; 16014924 |
| | DC-SIGN and IDO | 15579280; 15452205 |
| | SOCS1 | 16381597 |
| | hMR | 15047828 |
| HIV nuclear import/genome integration | Importin 7, Nup 98 | 12853482; 16103209; 15207818 |
| | LEDGF/p75 | 16439544 |
| | hCycT1 | 15913611 |
| | DBR1 | 16232320 |
| | PARP-1 | 15280503; 16002043 |
| HIV replication | Arp2/3 | 15385624 |
| | CDKN1A/p21 | 15767448 |
| | CyPA | 15254276 |
| | hSpt5, Cdk9, Cdk2 | 15620346; 15780141; 16085226 |
| | DHS | 15630446 |
| HIV packaging and budding | hRIP, Sam68 | 15749819; 15701759 |
| | LysRS | 12941890 |
| | Rab9 | 16140752 |
| | Tsg101 protein | 11595185 |
| | LIP5 | 15644320 |
| Others | p38 | 15928037 |
| | Furin 1, Furin 2, Pak1, Pak3 | 16352537 |
| | Cul5-E3 | 16014920 |

[a] While most relevant literatures published in 2005 and 2006 are listed here, only a selected representative papers are referenced for early studies

[b] For cellular targets involved in viral entry into cells, each specific target was references in the text part. This table covered more related studies in this category as a group except DC-SIGN and IDO, SOCS1, hMR are individually referenced

Varicellar zoster virus [13–29]. Early studies employing siRNA against viral infections mainly focused on viral genes. Nevertheless, the realization that there are divergent mechanisms for developing viral immunization from RNA interference led instead to the evaluation of cellular targets of viral infections. The identification of the physiological and pathological roles of cellular proteins that facilitate viral infections not only directly provides candidate targets for RNA interference, but also offers drug development targets for strategies such as antisense RNA, antibody, or other small molecules. As listed in Table 1, a large assortment of cellular proteins involved in viral entry into cells, viral RNA integration into the human genome, viral replication, viral packaging, and viral release, have been tested as potential targets for efficiently inhibiting HIV infection by siRNA technology (Fig. 1).

The identification of large number of novel cellular targets involved in HIV infections is greatly appreciated the development of different sized siRNA libraries (Table 2). The growing availability of siRNA libraries for high-throughput screening will accelerate the discovery of additional cellular targets of HIV infection. For example, Nguyen et al. recently performed a subgenomic screen using an siRNA library targeting 500 genes in HeLaCD4βpal cells challenged with HIV type IIIb [30]. In addition to confirming the involvement of furin in HIV replication, this screen revealed two novel cellular targets, Pak1 and Pak3. Overexpression of constitutively active Pak1 enhanced HIV IIIb infectivity while the knockdown
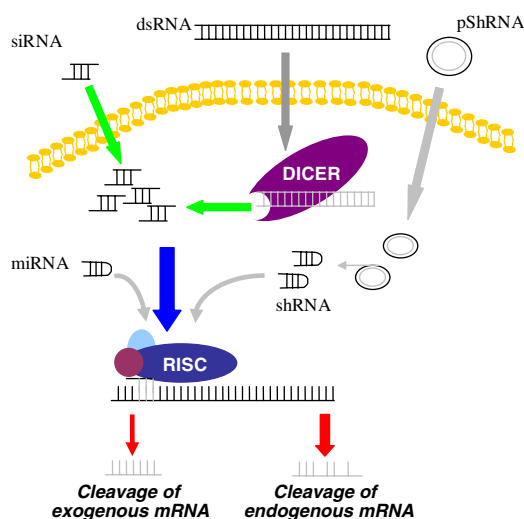


**Fig. 1** Diagram illustrating the mechanisms of RNAi in breaking down the mRNA of viral and cellular targets. The siRNA, double strands RNA, and shRNA need to penetrate the cell membrane before they are able to perform RNA interference. The RNA interference mediated by double strands RNA is DICER dependent, whereas the effects of exogenous siRNA and shRNA are DICER independent, which is similar to the mechanism of microRNA that cleaves RNA by RISC

of both targets decreased the amount of integrated HIV provirus. While the exact mechanisms for how these two targets help HIV replication are not currently known, HIV may utilize these Paks to enhance multiple stages of the viral life cycles.

Among cellular targets, the chemokine receptor, CCR5 is one of the few cellular exceptions that have been widely studied by different strategies including RNA interference. The main focus in the sections below is RNA interference against cellular targets of HIV infection (Fig. 2).

## Viral Responses to RNA Interference

RNA interference is a natural phenomenon occurring between virus and host during a virus invasion. Therefore, while RNA interference may sufficiently inhibit viral replication it rarely eliminates a viral infection. There are at least three known mechanisms employed by viruses to escape from RNA interference.

The first mechanism is silencing suppression and exists in both plant and animal viruses. By encoding silencing suppressors such as B2, NS1, VA1 RNA, and E3L, certain viruses can efficiently inhibit key components of the RNA silencing pathways [31–35]. Some suppressors can also regulate host gene expression. The second mechanism is silencing evasion. Silencing evasion could result from silencing-related ribonucleases that help to keep the viral genome away from siRNAs [36]. Another possibility for silencing evasion might be related to some viral genomes becoming intrinsically resistant to RNA degradation [37]. In contrast to the previous two mechanisms, the third mechanism called silencing immunizations requires a relatively short time adaptation and results in a rapid and widespread resistance mechanism against RNA interference. Although siRNA may be effective at the beginning, its effect can be progressively dampened by viral mutation at the target site. Alternatively, if a target site mutation is not tolerable for viral function the neighboring sequences can be mutated to change the secondary structure of the target region and thereby enable it to escape siRNA-triggered degradation [38–41].

## Bypassing the Hypermutability of HIV with Host Gene Knockdown

High mutation rate of HIV has been an obstacle in chemotherapy for AIDS. Notably, viruses employ mechanisms that can minimize or abolish the effects of siRNAs. These mechanisms include the suppression of RNA interference, evasion of RNA silencing, and immunity from or resistance to RNA silencing. Either the expression of certain viral

**Table 2** Methods for siRNA/shRNA gene transfer

| Method | SiRNA library | Specific siRNA | Cell type | PMID |
|---|---|---|---|---|
| Cationic lipid compacted, embryonic transfer | | Tested | Endothelial | Developmental dynamics 235:105 |
| Reverse chemical transfection | Possible | Tested | Cultured human cell | RNA (2005) 11:985 |
| Bacterial invation | Small to medium scaled | Tested | Mammalian cells | Nature methods 2(12):967 |
| Viral vector | Tested | Tested | Mammalian cells | Well established |
| Lipid mediated | Tested | Tested | Mammalian cells | Well established |
| LoxP-Cre system (shRNA) | Possible | Tested | Tissue specific knockdown | Genesis 44:252 (2006) |

genes or mutation of the silencing targets can enable viruses to replicate under RNA silencing pressure. The recognition of viral resistance to RNAi prompted strategies in evaluation of cellular co-factors required for HIV infection. In contrast to viral targets, RNAi against cellular targets may provide a more persistent inhibition to viral infection. In order to overcome the high mutation rate of HIV, recent efforts in screening new cellular targets of RNA interference show promises in viral replication. At the in vitro cultured cell level, many promising cellular targets pertinent to HIV infection have been validated. Further evaluation of these cellular targets by knockdown technologies may pave a new avenue for viral therapy as well as provide leads for the development of other small molecule therapeutic agents. Finally, RNA interference can be used to quickly screen the potential synergistic effect of knockdown of more than one cellular target in inhibiting HIV replication. This is important as it may substantially

minimize cellular toxicity and enhance its effects against HIV replication. In addition to different host genes in CD4 cells, targeting to different cellular genes at different cells may be of importance. For example, simultaneous knock-down of DCs express the DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) receptor in immature dendritic cells (DCs) and CCR5 is expected to work synergistically.

## RNA Interference Targeting Cellular Genes Involved in HIV Infection

A variety of different cellular targets that are involved in viral infection processes can be chosen. It should also be kept in mind that the cell targets discussed in each sub-category herein may have more than one role in viral infection. However, while a major advantage of targeting
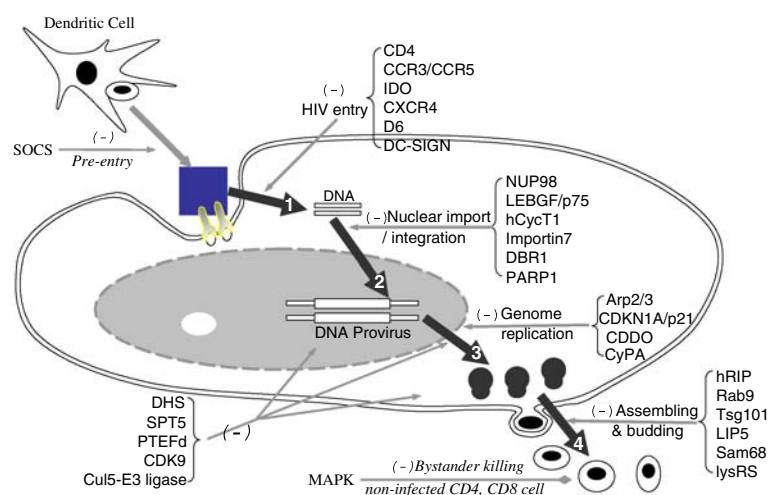


**Fig. 2** Cellular proteins evaluated by RNAi against specific steps of HIV life cycle. In addition to the cellular targets with known mechanisms inhibiting the four process of HIV infection as numerically marked, the inhibition of SOCS on pre-entry, the inhibition of bystander killing to non-infected CD4/CD8 cells, and some cellular targets with either more than one mechanisms or unknown mechanisms are also illustrated

cellular factors is its putative stable inhibitory effect, potential toxicity effects, such as cellular apoptosis due to the decrease of certain vital proteins, need to be extensively evaluated using in vivo studies since in vitro data may not be reliable regarding the potential toxicity issues. Considering its highly sequence-dependent toxicity, more cautions must be paid to RNAi related clinical trials such as testing a wider range of doses, a longer follow up, and a relatively larger sample included in phase I studies. These suggestions should be integrated into future clinical trials.

### Inhibition of HIV Entry

While some cellular proteins are crucial for viral RNA transcriptional replication, other cellular proteins take part in pre- or post-transcriptional aspects of the viral life cycle, such as entry into or budding out of host cells. As viruses are obligatory intracellular parasites, entry into the restricted range of cells limit their species and cell tropisms. The consequences of viral infection depend on how efficient viral genome transmits from infected to non-infected host cells. While some viruses only use one type of receptor to enter into cells, HIV-1 is a well-known example of viruses that need more than one type of cellular receptor in initiating or enhancing membrane fusion.

When targeting a variety of cell surface proteins, CD4, CCR5, CXCR4, and D6 have been well documented to effectively inhibit HIV-1 infection (Table 1). Using a genetic suppressor element technology, knockdown of 12 cell surface proteins identified 10 novel cellular targets with 60–90% inhibition of HIV-1 replication [42] (Table 1). These cell surface proteins represent novel targets for the development of therapeutics against HIV-1 infection and AIDS. In addition to CD4 cells, both microglia, the residential macrophages in the brain, and astrocytes are susceptible to HIV-1 infection. Unlike microglia that express and utilize CD4 and chemokine coreceptors CCR5 and CCR3 for HIV-1 infection, astrocytes fail to express CD4, instead, the cDNA for the human mannose receptor (hMR) was found to be essential for CD4-independent HIV-1 infectivity. Anti-hMR serum and hMR-specific siRNA blocked HIV-1 infection in human primary astrocytes [43].

Among the cellular targets, CXCR4 and CCR5 are the most extensively studied and a variety of strategies have been applied in developing specific therapeutic agents against those, including antibody, ribozyme, antisense RNA, and other compounds or compound cocktails [44, 45]. With the availability of siRNA technology, many groups have also evaluated the efficiency of siRNAs targeting these cell surface proteins. Martinez et al. demonstrated that the inhibitory effects of RNAi directed against CXCR4 was detectable 48 h after transfection of CXCR4 positive U87-CD4 positive cells [46]. Similar observations from Ji et al. [47], Lee et al. [48], and Anderson and Akkina [49] also confirmed that RNAi may be used to block HIV entry and replication. In addition to targeting CXCR4 and CCR5 separately, dual specific short-hairpin siRNA constructs containing an 8-nucleotide intervening spacer, targeted against either CXCR4 and CD4 or CCR5 and CXCR4 were also evaluated. Cleavage of the bispecific constructs to yield monospecific siRNAs was shown to occur in cell extracts [50].

During the early stages of HIV-1 infection, immature dendritic cells (DCs) work as the first line of defense against HIV infection. DCs express the DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) receptor and the T-cell suppressing factor indolamine-2,3-dioxygenase (IDO). These factors capture small amounts of HIV-1 found on mucosal surfaces and spread the viral infection to CD4(+) T cells. Arrighi et al. developed shRNA-expressing lentiviral vectors capable of conditionally suppressing DC-SIGN expression [51]. As expected, suppression of DC-SIGN expression inhibited the attachment of the HIV-1 gp120 envelope glycoprotein to DC-SIGN transfectants, as well as transfer of HIV-1 to target cells in trans. Epidemiological evidence has demonstrated the high risk of cocaine abuse and HIV infection. Recent experimental data show that cocaine up-regulates DC-SIGN among other genes, further supporting the therapeutic value of targeting DC-SIGN to inhibit HIV infection [52]. Moreover, DC-SIGN has already been employed in the development of other types of therapeutic agents (Table 1).

In addition to inhibiting HIV-1 transfer in trans, dendritic cells are also tested in an effort to induce anti-HIV-1 immunity by Song et al. [53]. By inhibiting suppressor of cytokine signaling (SOCS) 1, a key negative regulator of the JAK/STAT pathways, dendritic cells were more resistant to HIV Env-mediated suppressor and were capable of inducing memory HIV-Env-specific antibody and T-cell response. The potency of HIV DNA vaccination is significantly enhanced by co-immunization with SOCS1 siRNA expressor DNA. This first attempt to elicit HIV-specific T-cell and antibody responses by inhibiting a host's antigen presentation attenuator may open a new avenue in HIV vaccination.

### Inhibition of HIV Nuclear Import/Genome Integration

Like other lentiviruses, HIV-1 utilizes a nuclear import strategy to import HIV-1 cDNA and viral protein complexes through the nuclear pore complexes (NPC) formed by nucleoporin proteins (Nup). Following siRNA-mediated depletion of Nup98, Ebina et al. showed specific impairment of NPC structure and certain functions, including nuclear import of HIV-1 cDNA [54]. Fassati et al.

observed that importin 7, an import receptor for ribosomal proteins and histone H1, is involved in the nuclear import of purified HIV-1 intracellular reverse transcription complex (RTCs) in primary macrophages [55], and further studied the therapeutic potential of targeting importin 7 by small interfering RNA to inhibit HIV-1 infection. However, a similar study reported by Zielske and Stevenson concluded that importin 7 might be dispensable for infection in natural, non-dividing targets of HIV-1 and simian immunodeficiency virus such as primary macrophage and Hela cells [56]. Although many cellular proteins are crucial for HIV nuclear import, the co-factor effect of importin 7 is not conclusively validated. The conflicting data may partially depend on the cell types used, the dividing stage of the cells used, and the parameters monitored.

Efficient integration of HIV-1 into human genome requires the interaction between the transcriptional coactivator lens epithelium-derived growth factor (LEDGF/p75) and the HIV-1 integrase. Knockdown LEDGF/p75 by siRNA in HeLaP4 cells resulted in a three to fivefold inhibition of HIV-1 replication [57]. The extent of LEDGF/p75 knockdown is closely correlated with the reduction of HIV-1 replication, strongly suggesting the therapeutic impact of this cellular target in drug development. Although single-cycle infection analysis in cell lines led to questions about the overall importance of p75 in the viral life cycle [4, 8, 10–13], Llano et al. in a study with intensified RNA interference and dominant-negative protein approaches revealed an essential role for LEDGF/p75 in HIV integration [58]. The data from Llano et al. suggested that perturbing the p75-integrase interaction might have therapeutic potential [58].

Cellular factors crucial to RNA processing are also candidate targets for siRNA interference-mediated inhibition of viral infection. Human cyclin T1 (hCycT1) is a cellular factor essential for transcription of messenger and genomic RNAs from the long terminal repeat promoter of HIV-1 provirus. Intracellular expression of shRNA targeting hCycT1 produced a down-regulation of hCycT1 without causing apoptotic cell death. Therefore, targeting cellular factor hCycT1 by shRNAs may provide an attractive approach for genetic therapy of HIV-1 infection in the future [59].

Recently, Ye et al. hypothesized that HIV-1 might form a genomic RNA lariat and designed three siRNA molecules targeting the human RNA lariat de-branching enzyme (DBR1) [60]. Interestingly, despite reduction of DBR1 mRNA expression by 80%, cell viability was not affected while DBR1 knockdown led to significant decreases in viral cDNA and protein production. Thus, it seems that the DBR1 function may be needed to debranch a putative HIV-1 genomic RNA lariat prior to completion of reverse transcription.

An additional cellular protein facilitating HIV-1's integration into the human genome is poly(ADP-ribose)polymerase-1 (PARP-1). Kameoka et al. showed that the integration efficiency of the HIV-1 genome near alphoid DNA is significantly reduced in PARP-1 siRNA-transfected human cells [61]. However, the effect of PARP-1 on inhibiting HIV integration is not generally accepted as a co-factor. For example, Ariumi et al. examined the susceptibility to infection with wild-type HIV-1 and to transduction with a vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1-derived lentiviral vector of human cells stably expressing small interfering RNAs against ATM, ATR, and PARP-1, and observed viral integration normally occurred in these knockdown cells. Similarly, the VSV-G-pseudotyped HIV-1-based vector could effectively transduce ATM and PARP-1 knockout mouse cells as well as human cells deficient for DNA-PK [62].

Inhibition of HIV Genome Replication

In an initial step toward controlling the replication of HIV-1, targeting actin-polymerization mediated by the Arp2/3 complex has proven useful. Knockdown of expression of both acidic domains from Arp2/3 complex-binding proteins such as the Wiscott–Aldrich syndrome protein or cortactin inhibited HIV-1 infection [63].

HIV-1 infection was demonstrated to induce the transcriptional regulation of cellular genes, among which cyclin-dependent kinase inhibitor 1A (CDKN1A/p21) was the most prominently up-regulated gene. Treatment of macrophages with p21 antisense oligonucleotides or small interfering RNAs reduced HIV-1 infection. p21 is also a successful example in which oligonucleotide technologies identified a cellular target for development of other small molecule agents. In this case, the synthetic triterpenoid and peroxisome proliferator-activated receptor gamma ligand, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), which is known to influence p21 expression, was used to suppress viral replication [64].

Cyclophilin A (CyPA) is a cellular protein that is not essential for cell viability, but is required for HIV-1 multiplication. Using antisense U7 small nuclear RNAs (snRNAs) that disturb CyPA pre-mRNA splicing and short interfering RNAs (siRNAs) that target CyPA mRNA for degradation, Liu et al. observed reduced HIV-1 multiplication in the human T-cell line, CEM-SS [43]. Since the two types of antisense RNAs function by different mechanisms, combining the two approaches may result in a synergistic effect.

Application of more than one agent, or combined therapy, is recommended for HIV treatment. The RNA interference, particularly those targets host genes have some evident advantages over HIV resistance to conventional drugs. Recently, Hauber et al. identified that

inhibition of human deoxyhypusine synthase (DHS) by siRNA efficiently suppressed the retroviral replication [65]. This group further demonstrated that the inhibition of DHS by another small molecular compound CNI-1493 had effects against the replication of certain viral strains with high-level resistance to inhibitors of viral protease and reverse transcriptase, reinforcing the great potential of targeting cellular proteins by siRNA in HIV-1 infection.

Inhibition of HIV Assembling and Budding

The replication of HIV-1 and its transportation from nucleus to cytoplasm depend on the interaction of viral gene products and a variety of cellular proteins. Two of these cellular proteins, hRIP and Sam68 have been targeted in inhibiting HIV-1 infection. hRIP is required for the ability of HIV-1 Rev to correctly localize viral RNAs in the cytoplasm [66]. Ablation of hRIP activity by siRNA (or dominant-negative mutant) mislocalized Rev-directed to the nuclear periphery and could result in the loss of viral replication in human cell lines and primary macrophages [67]. Sam68 was initially identified as a 68 kDa Src-associated protein in mitosis [68, 69]. By expressing essentially full-length Sam68 antisense RNA, Li et al. obtained 80–86% reductions in HIV-1 replication by interfering HIV-1 exportation from the nucleus [70]. Recently, Modem produced similar inhibition on HIV-1 replication with siRNA targeting Sam68 [71].

HIV packaging and budding are complicated procedures that are currently poorly understood. During assembly, the major human tRNA(Lys) isoacceptors are selectively packaged into HIV-1, where they act as primers for reverse transcription. Guo et al. transfected 293T cells with HIV-1 proviral DNA and siRNA specific for LysRS and demonstrated reduced tRNA(Lys) packaging, reduced annealing to viral RNA, and reduced viral infectivity for the viruses produced from the cells transfected [72]. HIV assembly can be inhibited by blocking Rab9, a cellular protein with its effectors in facilitating vesicular transport by tethering donor vesicles to their respective target membranes. Using siRNA to silence Rab9 expression before viral infection, Murray et al. examined the role of Rab9 in the life cycles of HIV and several unrelated viruses [73]. They observed a wide spectrum of viral replication inhibition by silencing Rab9 expression including the enveloped Ebola, Marburg, and measles viruses in addition to HIV. Clearly, Rab9 is a significant cellular target for inhibiting diverse viruses due to its involvement in a late-endosome-to-plasma-membrane vesicular transport pathway important in viral assembly.

HIV-1 uses cellular machinery to bud from infected cells similar to other enveloped viruses. Tsg101 protein, which functions in vacuolar protein sorting (Vps), is required for HIV-1 budding. Depletion of cellular Tsg101 by siRNA arrests HIV-1 budding at a late stage while budding is rescued by reintroduction of Tsg101. This cellular target pathway was further validated using dominant negative mutant Vps4 proteins that inhibit vacuolar protein sorting and which also arrest HIV-1 and MLV budding. These observations suggest that retroviruses bud by appropriating cellular machinery normally used in the Vps pathway to form multivesicular bodies [74]. Cellular protein LIP5 is also required for HIV-1 budding. Results from Ward et al. showed that depletion of LIP5 by siRNA could decrease HIV-1 budding by 70% [75].

Other Potential Cellular Targets Against HIV Infection

For cells infected with herpes simplex virus, siRNAs designed to target viral gene expression were found to induce type I interferon synthesis through toll-like receptor 3 (TLR3) [20]. As the interferon effect is sequence independent and TLR3 is expressed in many cell types, whether this cellular target can be used for attenuating the clinical feature of AIDS is worth further evaluation.

In addition to directly killing infected cells, HIV has been reported to target non-infected CD4 and CD8 cells for destruction. This bystander killing is partially mediated by p38 mitogen-activated protein kinase (MAPK), which may contribute to the early onset of AIDS and the worsening of clinical manifestations in AIDS patients. p38 is also one of the few cellular targets have been promising in the development of chemical inhibitors [76].

Elevating the suppressed antiviral capability of HIV infected cells has shown some potential, evidenced by results from interference with Cul5-E3 ligase [77]. The human cytidine deaminase Apobec3F, a protein related to the previously recognized antiviral factor Apobec3G, has antiviral activity against HIV-1 that is suppressed by the viral protein Vif. Interference with Cul5-E3 ligase function by depletion of Cul5, through RNA interference or over-expression of Cul5 mutants, blocked the ability of HIV-1 Vif to suppress h-A3F. Developing inhibitors to disrupt the interaction between Vif and Cul5-E3 ligase could be therapeutically useful, allowing multiple host antiviral factors to suppress HIV-1.

## Current Limitations of and Research Focuses in Using RNAi Therapeutics

Significant advances have been made in the past two decades with regards to gene therapy using nucleotide related agents such as ribozyme, antisense oligonucleotides, and siRNA. However, there are three limitations that restrain

the clinical application of siRNA/shRNA in the treatment of HIV infections. These three issues are the toxicities of RNA interference due to off-target effects, the difficulties of using in vivo model to validate potential drug leads from in vitro screening, and the inefficiency of delivery of nucleotide-derived agents to target cells.

## Identification of Cellular Targets with More Inhibition on HIV Infection and Less Cytotoxicity

Cytotoxicity is one of the major obstacles preventing RNAi from being practical therapeutic agents. As a natural mechanism against viral infection, detection of viral RNA molecules triggers antiviral innate defense mechanisms including the induction of type I interferons and down regulation of viral and some cellular gene expression (In addition to the interferon response, siRNA or shRNA may also exert other non-specific effects through saturating cellular RNA machinery and thereby inhibiting the normal function of endogenous miRNAs. The third possible toxicity is the inhibition of non-target mRNAs by sequence complementarity. To increase the specificity and decrease the toxicity of siRNA/shRNA, appropriate design is crucial recommended as follows: (i) minimize homology to non-target mRNAs; (ii) avoid perfect dsRNA stretches of 11 bp; and (iii) minimize incorporation of the sense strand into RISC [14].

Better understanding of the mechanisms leading to the toxicity of therapeutic RNA interference is essential before RNAi can become a practical treatment for human diseases including HIV infection. Currently, an alternative solution to minimize the toxicity of RNA interference is to employ conditional expression strategies, and some reversible on/off switch or off/on switch systems controlled by doxycycline or ecdysone are already available over pol III and pol II promoters [78]. These strategies can avoid persistent toxicity to normal cells and allow the RNAi to be reversibly tunable in down-regulating gene expression related to cancer growth or viral replication.

Different cellular targets display varying thresholds for knockdown by RNA interference. For example, the human transcription elongation factor, SPT5, and the human mRNA capping enzyme are two proteins crucial for cellular RNA processing. Knockdown of hSpt5 did not significantly affect cell viability but siRNA-mediated silencing of human mRNA capping enzyme was lethal to mammalian cells [79]. Selective knockdown of hSpt5 led to significant decreases in Tat transactivation and concomitant inhibition of HIV-1 replication, indicating that hSpt5 was required for mediating Tat transactivation and HIV-1 replication. A similar phenomenon was observed with human positive transcription elongation factor, P-TEFb, a factor composed of two subunits, cyclin T1

(hCycT1) and Cdk9, that is involved in transcriptional regulation of cellular genes as well as HIV-1 mRNA. Chiu et al. showed that RNAi-mediated gene silencing of P-TEFb in HeLa cells was not lethal and inhibited Tat transactivation to activate elongation of RNA polymerase II and HIV-1 replication in host cells, suggesting that there is a critical threshold concentration of activated P-TEFb required for cell viability and HIV replication [80]. Similar to Cdk9, another cell cycle-dependent kinase Cdk2 is not essential in mammalian development but is crucial to the functionality of the HIV-1 Tat protein [81, 82].

The lack of convenient in vivo model of AIDS is a drawback in confirming initial in vitro screened co-factor of HIV infection. This is particularly obvious when discrepancies are reported about the same cellular target. One observation can differ from another due to assays employed, primary cells or cell lines used, cell culture conditions particularly growth factors in the media, and many other factors. Table 2 includes a list of well-confirmed cellular targets and some targets that are not generally accepted as a co-factor for HIV infection. The well-confirmed targets are usually applied in development of other types of therapeutic agents. Therefore, re-evaluation of initially screened cellular targets by RNA interference with other mechanisms such as using ribozyme or antisense oligonucleotides could be a remedy to the difficulty of using in vivo model in HIV research. Similarly, it is always more reliable to apply at least one strategy other than RNA interference to confirm any newly identified co-factor of HIV infection by siRNA/shRNA.

## Development and Optimization of In vivo Delivery System for Nucleotide-based Agents

Steady progress has been obtained in improving the delivery efficiencies of nucleotide-derived agents as shown in Table 2. A variety of methods, including protein-based, liposome-based, and viral-based transfections are widely used for in vitro studies. Different methods have their own advantages and disadvantages. The convenience is the unique feature of liposome for gene transfer in cultured cells. However, the cell type dependency and relative toxicity limited the in vivo application of liposome. Particularly, the application of lentiviral vectors allows the transfection of non-dividing cells to be possible both in vitro and in vivo. Recently, ter Brake et al. developed a lentiviral vector that can express three different shRNAs in one vector [83]. The combined expression results in a stronger inhibition of virus production. In contrast to the in vitro application, in vivo delivery of siRNA is still a challenge. An increased efficiency, more selection in cells targeted, and prolonged half-life of administrated therapeutic agents are expected to be further improved.

## Therapeutic Potential Provided by RNAi and its Future Perspectives in AIDS Therapy

It is not realistic to expect a cure for HIV infection in the near future simply depending on RNA interference. Instead, we are only able to postpone the development of AIDS from HIV infection based on the viral targets and cellular targets we have thus far. With the recognition of cellular elements that have distinguishable effects on cell function and viral replication, such as the CCR5 wild-type and mutant, we can expect the identification of one or more crucial cellular targets which HIV replication is highly dependent upon but cell division is less dependent or not dependent at all. As discussed earlier, greater efforts in identifying more cellular targets specifically required by HIV replication are one of the main topics in near future research of RNA interference.

RNA interference is one of the members of ribosomal pharmacology that also covers ribozyme and antisense RNA. Theoretically, there is no doubt that siRNAs can be both a tool in antiviral studies and provide screening leads for antiviral drugs. Although no siRNA-based drug has yet been FDA approved, Vitvarene, an antisense drug against CMV retinitis, has been FDA-approved and successfully marketed. It is reasonable to speculate that many more RNA drugs treating human disease including viral infection, either antisense RNA or siRNA-based, will be approved in the future. Presently, there are only a limited number of drugs or leads under clinical trials, however, RNAi is so far the most powerful tool in identifying viral or cellular targets possessing therapeutic potentials. These targets help to better understand the biology of HIV replication within human cells, and provide therapeutic targets for the development of chemically synthesized small molecular drugs. The latter allows maximization of the advantages of chemical synthesized small drugs: easy to deliver and longer in vivo half-life. Therefore, significant shortening of target identification by RNA interference has great impact in the fight against HIV infection. Another major advantage of using siRNA/shRNA to treat viral infections such as HIV infection is the relative ease of design for the candidate leads, rapid evaluation for in vitro testing, and most importantly the large collection of siRNA/shRNA libraries for high throughput screening of novel targets as shown in Table 2. siRNA/shRNA has precious value in fighting HIV infection as RNA interference facilitates the understanding of the biology of HIV replication, offers efficient identification of therapeutic targets, and can be drug leads themselves. Among these multiple impacts, host genes required for HIV replication may play a crucial role in the efforts of obtaining an optional control for HIV infection, such as the combination of drugs of several cellular targets or the combination of siRNA/shRNA drugs together with other drugs against cellular or cellular and viral targets.

The recognition of RNA interference has caused a revolutionary change in genetic research and in drug target screening. The utility of RNAi for target discovery has been well established both at transcriptional and post-transcriptional levels. For example, there is no difficulty in applying RNA interference to the screening of some particular targets such as proteins lacking small molecule binding activities or non-coding RNAs. As discussed earlier, one of the immediate biomedical benefits of using siRNA/shRNA is the significant shortening of time required for drug target screening. However, for siRNA/shRNA to be therapeutic agents in treating viral infection, much has been done to overcome the aforementioned restraints, particularly the inefficient delivery of nucleotide-derived agents in vivo. Substantial progress in improving the efficiency of siRNA/shRNA delivery will also benefit other nucleotide-based agents such as ribozyme and antisense oligonucleotides.

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