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Interfering antiviral immunity: application, subversion, hope?

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RNA interference (RNAi), initially recognized as a natural antiviral mechanism in plants, has rapidly emerged as an invaluable tool to suppress gene expression in a sequence-specific manner in all organisms, including mammals. Its potential to inhibit the replication of a variety of viruses has been demonstrated *in vitro* and *in vivo* in mouse and monkey models. These results have generated profound interest in the use of this technology as a potential treatment strategy for viral infections for which vaccines and drugs are unavailable or inadequate. In this review, we discuss the progress made within the past 2–3 years towards harnessing the potential of RNAi for clinical application in viral infections and the hurdles that have yet to be overcome.

**Introduction**

Conventionally, only specialized cells of the immune system and their secreted products are thought to be involved in protecting the body from foreign invaders such as viruses. However, in recent years, a new type of genomic immunity mediated by RNA interference (RNAi) has emerged and has sparked intense interest as a potential antiviral treatment strategy for a variety of diseases, including viral infections, cancer and degenerative diseases [1–4]. RNAi was first recognized as a naturally occurring anti-viral defense mechanism in plants. In RNAi, long double-stranded (ds) RNA generated during viral infection is cleaved by an enzyme termed Dicer into short, 21–23 nucleotide (nt) dsRNA molecules termed small interfering (si)RNAs that mediate sequence-specific gene silencing [5,6]. The siRNA associates with a protein complex called the RNA-induced silencing complex (RISC) (Figure 1), following which the sense strand is cleaved by the enzyme Argonaute 2 (Ago2) [7]. The antisense strand then guides the RISC to the corresponding messenger (m)RNA by sequence homology, and the Ago2 nuclease cuts the mRNA, resulting in specific gene silencing. In the context of RNA viruses, siRNAs can be designed to degrade not only viral mRNA but also the negative sense viral genomic RNA and the complimentary RNA that serves as a template for new genomic RNA synthesis [8]. Although RNAi is a natural phenomenon in plants and worms, long dsRNA induces an interferon response in mammalian cells resulting in non-specific global suppression of protein synthesis and cell death [9].

A landmark development in the field occurred with the discovery that the introduction of 21-nt-long synthetic RNA resembling the Dicer-processed siRNA into mammalian cells induces sequence-specific gene silencing without evoking the interferon response [10]. Since then, RNAi has been widely used as a quick reverse-genetics approach for gene-function analysis and for ablating specific genes for therapeutic purposes. The exquisite sequence specificity and high potency of RNAi makes it an attractive gene-silencing approach [6]. RNAi was found to be 1000-fold more effective on a molar basis than antisense oligonucleotides [11]. This is probably owing to the autocatalytic effect of RNAi whereby a single siRNA molecule is reused for cleaving many target mRNA molecules [10,12]. RNAi can be induced by the introduction of synthetic siRNA or by intracellular generation from vector-driven expression of precursor short hairpin (sh) RNAs (Box 1). The optimal design of siRNA or shRNA is required for the potent induction of RNAi (Box 2).

**RNAi for suppressing viral infections**

Although RNAi is an integral component of the innate immune response to viruses in plants, whether the same is true in mammals is unclear. However, the recently described virus-encoded counter-defense strategies, such as suppressors of RNAi and microRNAs, suggest a long-standing interaction of viruses with the RNAi machinery (Box 3). Given the natural antiviral role of RNAi in plants and its induction in mammals by introduced siRNA, the phenomenon has generated great enthusiasm as a potential antiviral treatment strategy [1,2]. Several viruses with widely differing replication cycles have been inhibited *in vitro* by targeting viral and cellular genes involved in the viral life cycle (Figure 2). These include: the positive-stranded RNA viruses, including polio, West Nile, dengue and foot and mouth disease (FMD) viruses; the negative-stranded RNA viruses, including respiratory syncytial virus (RSV) and influenza virus; the double-stranded RNA rotavirus; HIV lentivirus; and DNA viruses such as the polyoma virus, papilloma virus and herpes simplex virus (HSV). However, the replicative characteristics of certain viruses can protect them from RNAi. For example, although siRNAs can inhibit progeny virus production, the genomic RNAs of RSV, hepatitis delta virus and rotavirus are resistant to RNAi owing to tight...
RNA interference. RNA interference can be initiated in cells by the introduction of synthetic double stranded siRNA or plasmid or viral vectors encoding shRNA. The shRNA is transcribed in the nucleus and exported to the cytoplasm, where it is processed into siRNA by Dicer or, possibly, another ribonuclease. In the cytoplasm, the siRNA associates with the RISC complex consisting of several proteins, which in human cells include Dicer, argonaute 2 (Ago-2), HIV-1 transactivating response RNA-binding protein (TRBP), protein activator of protein kinase R (PACT) and, possibly, other proteins unidentified to date [50]. The sense (passenger) strand of the siRNA is then cleaved by Ago-2 within the active RISC [7]. The passenger strand can also be removed, albeit at a slower rate, by a cleavage-independent 'bypass' mechanism used for microRNA processing [7]. Because the exact sequence of the molecular interactions involved in RISC activation is unknown, the process is shown in a dotted box and the siRNA guide strand is shown as curved to indicate its directional loading into the RISC. The anti-sense (guide) strand associated with the mature RISC guides the complex to the corresponding mRNA because of sequence homology, and the same Ago-2 nuclease then cuts the target mRNA at a position corresponding to nt 10–11 from the 5'-end of the anti-sense guide strand. The cleaved mRNA is rapidly degraded resulting in gene silencing.

Box 1. Synthetic siRNA versus vector-driven shRNA
RNAi can be induced by synthetic siRNA or by vector-driven expression of shRNA. In the second method, siRNA sequence followed by a ~9nt loop and a reverse complement of the siRNA sequence is cloned in DNA or viral vectors to express endogenously the shRNA, which is processed in the cytoplasm to siRNA. Whereas synthetic siRNA is introduced into cells by transfection, shRNA can be introduced by transfection of a DNA vector or transduction through viral vectors. Non-replicating, recombinant viral vectors are commonly used for shRNA expression because of ease of delivery, particularly in difficult-to-transfect primary cells. Adenoviruses and adeno-associated viruses have been used as vectors but lentiviral vectors are generally preferred because they infect actively dividing, and resting and differentiated cells such as stem cells, macrophages and neurons. Because the viral DNA is incorporated in the host genome, the main advantage of this method is the long-term expression of shRNAs and gene silencing. In fact, knockdown persisted for at least six months in the mouse brain following transduction with a shRNA-expressing vector [51]. Although viral vectors deliver shRNA efficiently, they have several disadvantages, including the vector-induced immune response and possible toxic effects of long-term RNAi induction. Moreover, retroviral integration into the host genome also enhances the risk of insertional mutagenesis, exemplified by the development of leukemia in patients undergoing retroviral-based gene therapy for severe combined immunodeficiency [49]. By contrast, synthetic siRNA, similarly to drug treatment, provides a way to achieve transient gene silencing without the risks of insertional mutagenesis, immune response induction or the toxic effects of long-term RNAi induction. The major challenge is its delivery to cells in vivo. Also, because siRNA becomes diluted by cell division, the silencing effect generally fades after 4–5 days in dividing cells. However, in non-dividing cells such as macrophages and neurons, siRNA silencing has been observed for at least 3 weeks [38,52]. Thus, whereas siRNA seems to be ideal for situations such as acute viral infection, shRNA could be useful for treating chronic viral infections and cancer. shRNA might also be useful to generate cells resistant to infection by transducing stem cells with shRNA-encoding vectors.
Box 2. Recent advances in siRNA and shRNA design

Until recently, the design of siRNA was based on selecting a 19-bp unique sequence with ~45–55% G:C content immediately following the nucleotides AA or NA (N represents any nucleotide) within the target gene, and incorporating a 5’ phosphate and 2-nt 3’ overhangs [10]. The chances of making successful hits in this essentially trial-and-error process required testing multiple target sites for each gene. However, based on analyses of the biochemical properties of a large number of highly effective siRNAs, more-stringent algorithms have now been developed for predicting functional siRNAs [8]. The thermodynamic stability at the 5’ end of the antisense strand has emerged as a crucial criterion for siRNA effectiveness [53,54]. A low internal stability at the 5’ end enables proper directional loading of siRNA to ensure stable incorporation of the antisense instead of the ineffective sense strand into the RISC. This increases the potency of siRNA almost a 100-fold, and, by reducing the amounts of siRNA needed for silencing, it also minimizes off-target silencing. Lower thermostability can also be ensured by incorporating mismatches in the sense strand at nucleotides complementary to positions 2–4 of the 5’ end in the antisense strand. Because the antisense strand is still unaltered in sequence, target specificity is not compromised. Alternatively, guanosines can be replaced with inosines in the first 4 positions to give I:C base pairs that are similar in energy to A:U base pairs, to increase the propensity of this end to fray. Low thermodynamic stability in the mRNA cleavage region is also important to promote the release of the RISC–antisense complex for multiple rounds of activity. Using a 27–29 nt siRNA instead of the conventional 21-nt siRNA increases the potency by 10–100-fold without inducing an interferon response [55]. This could be because the longer siRNA is processed by Dicer to generate the optimal siRNA endogenously.

The Pol III promoters, such as U6, H1 and TRNA, are commonly used to drive shRNA expression because they provide an efficient mechanism to generate small RNA transcripts. However, it has recently been shown that inserting the shRNA sequence into the backbone of a miRNA (e.g. Mir-30) at the stem increases the shRNA potency enormously, even at the level of single-copy integration [56]. Because the Pol II cytomegalovirus (CMV) promoter is used to drive this longer shRNA, this system also enables multicistronic expression of multiple shRNAs.

shielding by proteins or sequestration in membranous compartments [13,14]. Despite success in vitro, many hurdles need to be overcome before using RNAi to counteract virus infections in vivo. One major bottleneck is the delivery of siRNAs and shRNAs to appropriate cell types in vivo but significant progress has been made in the past 2–3 years. Another limitation is the lack of appropriate animal models for many human viruses. Nevertheless, studies in mice have proved invaluable for testing the in vivo efficacy for some viruses. Data obtained from these initial investigations provide a glimpse of the successes and challenges that can be expected in a clinical setting for specific viruses in terms of RNAi design and delivery. In the next subsections, we describe some of the viral diseases for which substantial progress has been made in moving RNAi towards therapy.

Viral hepatitis
Although mice are not susceptible to hepatitis viruses, transfection with plasmids containing the viral genome recapitulates many steps in the viral life cycle, including DNA replication and expression of the core and surface antigens. In one of the first demonstrations of RNAi effectiveness in vivo, McCaffrey et al. injected the pTHBV2 plasmid encoding the hepatitis B virus (HBV) genome alone or with a plasmid encoding anti-HBV shRNAs by hydrodynamic intravenous injection (DNA was rapidly injected intravenously in 10–15 seconds in a large volume of >1 ml) [15]. This resulted in a substantial knockdown of HBV transcription in the liver and also resulted in >90% reduction in serum hepatitis B surface antigen (HBsAg) levels and viral core antigen expression by liver cells. Hydrodynamic injection is not feasible in humans because it entails the injection of almost the whole blood volume. However, Morrissey et al. showed that regular low-volume intravenous injection of a chemically modified siRNA (containing a phosphorothioate backbone and 2’-fluoro and 2’-O-methyl substitutions to render the siRNA nuclease-resistant) can also significantly reduce serum DNA and HBsAg levels in mice [16]. Chemically modified siRNAs have also been encapsulated in lipid nanoparticles for intravenous delivery [17]. This resulted in extending the serum half-life of siRNA from ~2 min to 6.5 h and reduced the siRNA amounts required for a comparable reduction in viral DNA and serum HBsAg from 30

Box 3. Manipulation of RNAi pathways by viruses

RNAi is used as a natural antiviral defense mechanism in plants, and plant viruses have also developed mechanisms to evade RNAi [57]. Although it is unknown whether RNAi is induced naturally during viral infection in mammals, recent studies suggest that mammalian viruses can also suppress RNAi [58,59]. Nodamura virus encodes a protein called B2 that interferes with Dicer function and the incorporation of siRNA into RISC [60]. Similarly, the E3L protein of vaccinia virus and the nonstructural protein NS1 of influenza virus can suppress RNAi by sequestering dsRNA [61,62]. The nonstructural protein NSs of La Crosse bunyavirus and HIV-1 tat protein also suppress RNAi [63,64].

In addition to siRNAs, another class of small RNAs called miRNAs also use the RNAi pathway [65]. In contrast to siRNAs, miRNAs are cellular gene products and regulate large numbers of genes. Many viruses have developed mechanisms to evade RNAi in plants, worms, and mammals. In fact, the altered development of T and B lymphocytes has been noted in specific miRNA-knockout hematopoietic stem cells and in Dicer-knockout mice [66,67].

Recently, viruses have also been found to encode their own miRNA and manipulate host miRNAs. Epstein-Barr virus encodes a miRNA BART2, which has been predicted to target several cellular genes [88]. Similarly, human cytomegalovirus encodes at least five miRNAs, and the Kaposi’s sarcoma-associated herpesvirus expresses 11 miRNAs that can target host genes [69]. The exact function of these viral miRNAs has yet to be elucidated. However, the SV-40 encoded miRNA downregulates SV-40 T antigens on infected tumor cells, making them less susceptible for cytotoxic T-cell recognition, providing the virus a way to evade host immunity [70].

Viruses can also use different methods for subverting host miRNAs for their own purposes. The liver-specific miRNA miR-122 binds to the 5’ end of the hepatitis C viral genome to greatly augment viral replication, and miR-122 inactivation abolishes viral replication [71]. By contrast, another host miRNA, miR-32, targets the retrovirus primate foamy virus-1 genome to repress viral replication, and the viral Tas protein suppresses the RNAi pathway to overcome miR-32 action [72]. A miRNA encoded within the HIV nef gene that can regulate viral transcription has also been documented [73].
to 3 mg/kg. Moreover, the reduction in serum HBV DNA could be sustained for 6 weeks simply by weekly siRNA treatment, showing the feasibility of using siRNA treatment for a chronic disease such as HBV infection.

**Influenza virus**

Influenza virus (IAV) is the major cause of respiratory infections worldwide. Two groups have recently used RNAi to suppress IAV infection in mice. By targeting a conserved sequence in the viral nucleoprotein and acid polymerase genes, Tompkins et al. prevented infection with multiple isolates of IAV, including the virulent avian H5N1 strain [18]. For delivery to the lungs, they injected siRNA by hydrodynamic intravenous injection combined with intranasal administration of siRNA complexed with the transfection reagent oligofectamine. Similarly, Ge et al. achieved
efficient lung delivery following regular intravenous injection of siRNA- or shRNA-encoding DNA vector by complexing with the cationic polymer polyethyleneimine (PEI) [19]. This treatment resulted in a 1–2 log reduction in viral titers even when administered 24 h post-infection. Moreover, the intranasal administration of shRNA vector with the surfactant Infasurf® or of siRNA complexed with PEI, were also effective, providing an intranasal approach to treat respiratory illnesses.

Respiratory syncytial virus
RSV is another major respiratory pathogen that causes epidemics of respiratory illness with bronchiolitis and pneumonia. Two studies have shown that siRNAs can be used effectively for prophylaxis and treatment of RSV infection. The viral nonstructural protein NS1 suppresses type I interferon production in host cells, and NS1 deletion mutants are avirulent in vitro and in vivo. Transfecting human dendritic cells with a plasmid encoding shRNA to suppress RSV NS-1 resulted in the efficient induction of interferon and interferon-induced genes following RSV infection [20]. To test its antiviral effects in vivo, the vector was complexed in a chitosan polymeric nanoparticle. Intranasal instillation of the nanoparticle was harmless in mice and substantially reduced viral titers and virus-induced pathology when administered 2 days before infection. Importantly, it also reduced lung inflammation and viral titers by nearly 2 logs even when administered 2 days after infection. Similar results were also obtained by Bitko et al. [21]. In this report, the authors used a synthetic siRNA targeting the viral P protein, an essential component of viral RNA polymerase. When administered intranasally with the TransIT® transfection reagent, the siRNA substantially suppressed virus replication and lung pathology. This effect was also seen, although to a lesser extent, when the siRNA was administered 2–3 days after viral infection. Interestingly, the intranasal application of naked siRNA without a transfection reagent was also effective (80% activity). Similarly, a siRNA targeting the P protein of parainfluenza virus was also able to suppress viral replication and virus-induced pathology [21].

SARS corona virus
An epidemic caused by the recently emerged respiratory viral pathogen SARS corona virus (SCV) attracted worldwide attention because of the high degree of morbidity and mortality it carried. A rhesus macaque monkey model has been developed for this virus, in which intranasal instillation of the PUMC01 strain of SCV results in a disease that is similar to the human disease [22]. Li et al. used this system to test siRNA as a potential therapy for SCV [22]. siRNAs were used to target the SCV genome at the Spike protein coding region. Because the lipid-based transfection reagents PEI and TransIT TKOR® used as siRNA carriers in previous studies might not be acceptable for human treatment owing to potential toxicity, the authors in this study used Infasurf® or 5% D-glucose in water (D5W) for siRNA delivery. Infasurf® (a naturally occurring lung surfactant protein) and D5W are nontoxic and are currently in clinical use. D5W was 3–4 fold more effective than Infasurf® in delivering siRNA to the lungs. For testing the efficacy in monkeys, 10 mg/kg of siRNA was intranasally instilled in 3 ml of D5W solution. This treatment substantially reduced clinical symptoms, lung pathology and viral burden. Although the inhibitory effect was maximal when the siRNA was administered 4h before or with viral challenge, disease mitigation was seen even when it was given 24h after viral challenge. Thus, this study demonstrated for the first time the considerable antiviral potential of RNAi in a non-human primate model using clinically acceptable carriers for siRNA delivery.

Herpes simplex virus
siRNA has also been used as a potential topical microbicid [23]. Intravaginal application of an anti-green-fluorescent-protein (GFP) siRNA mixed with oligofectamine in GFP-transgenic mice resulted in the loss of GFP expression throughout the vagina and cervix but not in distant organs, such as the liver. Importantly, the topical application of siRNAs targeting the essential HSV-2 genes UL27 (envelope glycoprotein B) and UL29 (a DNA-binding protein) before viral infection reduced mortality by 60% and substantially reduced viral shedding from the vagina. Combinations of siRNAs were also effective when administered 3 and 6 h after infection. This study highlights the feasibility of using a similar approach for other sexually transmitted diseases, including HIV-1.

Encephalitogenic flaviviruses
Japanese encephalitis (JE) and West Nile (WN) viruses can cause a devastating neurological illness. Two studies have used RNAi to suppress these viruses in mouse models. Bai et al. injected a siRNA targeting the viral envelope gene hydrodynamically 24h before an intraperitoneal WNV challenge and observed a 40% increase in survival [24]. Kumar et al. targeted conserved regions in the viral envelope genes of JEV and WNV and administered siRNA through a lentiviral vector or as synthetic siRNA complexed with the cationic lipid JetSI/Dope to enable siRNA delivery to neuronal cells [25]. A single intracranial treatment with lentiviral vector or synthetic siRNA was sufficient to provide almost complete protection from fatality. siRNA treatment given 18 h after infection was also effective but the treatment failed at later time points. Significantly, by targeting a sequence that is highly conserved in JEV and WNV, they achieved near-complete protection against fatal encephalitis induced by either JEV or WNV. This offers the possibility of using siRNA as a broad-spectrum antiviral agent to suppress related viruses across species.

Foot and mouth disease
FMD is a highly contagious and economically devastating disease of domestic animals. Chen et al. used a plasmid vector encoding a shRNA targeting the viral VP-1 gene in a suckling mouse model [26]. The subcutaneous injection of 50 μg of plasmid DNA 6h before FMD challenge resulted in 75% survival, compared with 100% mortality in control-DNA-injected mice. However, DNA vector injection at the time of viral challenge or increasing the challenge virus dose reduced the protection considerably.
**HIV**

Interest in RNAi as an alternative antiviral approach has been particularly strong for HIV-1 because of the problems of drug resistance, toxicity and the cost of highly active antiretroviral therapy (HAART). Several investigators have used RNAi to suppress HIV in cell lines and primary cells [27–29]. However, given the specificity of RNAi, the propensity of the virus to mutate can pose a serious challenge for therapeutic use. *In vitro* studies have documented the generation of RNAi-escape mutants in long-term cultures [30,31]. Nevertheless, recent studies suggest that although the homology requirement for RNAi is stringent for the crucial central residues, there is some tolerance for peripheral nucleotide changes [32]. Moreover, because RNAi requires only short stretches of homology, it is possible to target one or more regions which are highly conserved because of their essential structural and functional roles. Several of these conserved target sites have been identified [33–36]. By targeting a highly conserved vif sequence, Lee et al. protected CD4 T cells from all HIV clades, including multiple isolates of clade B, which is prevalent in the West [33].

Host genes important for HIV replication, including the viral receptor CD4 and the co-receptors CCR5 and CXCR4, have also been targeted to avoid viral escape. Synthetic and lentivirally-expressed siRNAs have been used to prevent HIV entry by silencing CCR5 [37,38]. CCR5 is the favored target because a 32 base pair (bp) deletion of the gene is known to be harmless and confers resistance to HIV infection [39]. Using a combination of siRNAs targeting conserved viral sequences and host genes important in the viral life cycle might be the optimal therapeutic approach, akin to antiretroviral drug cocktails. In another study, RNAi has been combined with other gene therapy approaches in a single lentiviral vector. The lentivirus was engineered to encode a shRNA targeting HIV-1 rev/tat mRNA, a short RNA homologous to the viral transactivation response region (TAR) to act as a decoy for TAR binding, and a ribozyme targeting the host CCR5 gene. The approach might be a pragmatic way for achieving the stable long-term suppression of HIV replication because each of these therapeutic RNAs targets a different gene product and blocks HIV replication by a distinct mechanism [40].

A novel approach has also been used for targeted siRNA delivery to infected T cells. Here, a chimeric recombinant protein consisting of a single-chain antibody to HIV-1 gp120 fused to the highly positively charged protamine was able to bind to siRNA (by charge interaction) and deliver it specifically to HIV-infected CD4+ T cells [41]. Moreover, when administered with bound anti-tumor siRNAs, it specifically targeted and cleared gp120-expressing experimental tumors in mice.

Another approach is to generate HIV-resistant progeny T cells and macrophages by transducing hematopoietic stem cells (HSC). The feasibility of this approach was shown in the severe combined immunodeficient (SCID) mouse–human chimeric model by transplanting human CD34+ hematopoietic stem cells transduced with a lentivirus expressing an anti-HIV shRNA [42,43].

**Potential limitations of RNAi therapy**

RNAi has already entered Phase I clinical trials for macular degeneration and RSV infection. However, there are several hurdles that must be overcome for routine therapeutic use. A significant limitation for its use in viral infections is the natural sequence differences that exist amongst the various serotypes and strains within a given viral species. Sequence divergence can also occur because of the accumulation of mutations during viral replication or the active generation of escape mutants [2]. Selecting conserved sequences where the virus is averse to mutate and using a combination of several viral and feasible cellular targets could overcome this limitation. Even so, target sequences based on the data available for the few sequenced viruses might not ensure effectiveness against field strains of virus. Thus, testing multiple field isolates *in vivo* might be necessary.

Although substantial progress has been made, more studies are required to achieve the effective and nontoxic delivery of siRNA and shRNA *in vivo*. Toxicity can be a problem for *in vivo* use, particularly when carriers such as transfection reagents are used for delivery. For example, although PEI has been used in one study in mice [19], several reports suggest that it induces toxic effects that might result in the death of the animals [21,22,44]. Thus, greater emphasis should be given to developing clinically acceptable carriers for systemic delivery. The encapsulation of siRNA within nontoxic nanoparticles or liposomes might enhance stability and simultaneously avoid toxicity. Combining a targeted delivery approach, using antibodies or receptor ligands, the introduction of chemical modifications in the siRNA, and the encapsulation of siRNA within nanoparticles or liposomes could be the ideal way to improve systemic delivery and reduce siRNA requirements. Delivery to the brain is still a challenge because of the blood–brain barrier. However, a recent study used transferrin receptor antibody-coated immunoliposomes to overcome this barrier [45].

Another issue is the induction of interferon and the associated inflammatory effects. In fact, toll-like receptor (TLR) recognition of RNA by plasmacytoid dendritic cells seems to be a major mechanism of interferon induction [46], and the administration of naked siRNA might induce interferon. However, certain motifs within the RNA seem to be crucial for interferon induction; avoiding such motifs or encapsulating siRNA within nanoparticles could reduce this risk [17,47]. A better understanding of the immunostimulatory motifs within the RNA that are needed for TLR activation might help to avoid non-specific immune activation. However, because most viral infections induce interferon and inflammation, this might not be a major limitation for antiviral therapy. The inadvertent targeting of host genes, observed in some *in vitro* studies [48], is another issue that needs more-thorough investigation. Although no grossly observable side effects have been reported *in vivo*, off-target effects might not necessarily manifest as symptoms in animals but could be unacceptable for human therapy. Also, because the RNAi machinery is used in mammalian cells to regulate cellular gene expression by microRNAs (miRNAs), the effect of the exogenously introduced siRNA on miRNA functioning...
should be investigated. Another concern is the effect of possible subversion of the RNAi machinery by viral proteins or virally encoded microRNAs (Box 3).

Safety is an also an issue in using shRNA delivery through viral vectors because of the possibility of insertional mutagenesis and malignant transformation [49]. Another concern is that the effect of long-term siRNA induction is unknown. Inducible systems for the controlled expression of siRNA from plasmid or viral vectors could offer hope in this regard.

Summary
RNAi has tremendous therapeutic potential in viral infections. The major hurdles of delivery to the appropriate cells necessary for its in vivo use as a therapeutic agent are being rapidly overcome and siRNA therapy is already being tested in clinical trials. Future studies should undoubtedly focus on further refining in vivo delivery methods, and minimizing off-target effects and the emergence of escape mutants in vivo. Optimizing siRNA design, delivery methods and delivery schedules to achieve sustained siRNA levels in the target cells should be emphasized. With these investigations and further advancement in the understanding of the endogenous RNAi mechanism, the next few years should prove to be an exciting time to discover whether RNAi will become a viable approach to treat viral infections in humans, particularly after the appearance of clinical symptoms.

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