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The silent treatment: RNAi as a defense against virus infection in mammals

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RNA interference (RNAi) is a mechanism for sequence-specific gene silencing guided by double-stranded RNA. In plants and insects it is well established that RNAi is instrumental in the response to viral infections; whether RNAi has a similar function in mammals is under intense investigation. Recent studies to address this question have identified some unanticipated interactions between the RNAi machinery and mammalian viruses. Furthermore, introduction of virus-specific small interfering RNAs (siRNAs) into cells, thus programming the RNAi machinery to target viruses, is an effective therapeutic approach to inhibit virus replication *in vitro* and in animal models. Although several issues remain to be addressed, such as delivery and viral escape, these findings hold tremendous potential for the development of RNAi-based antiviral therapeutics.

Introduction

The term RNA silencing refers to several gene-silencing processes triggered by double-stranded RNA (dsRNA): these are highly specific and conserved in virtually all organisms. RNA silencing mechanisms are initiated by dsRNA, which is cleaved by the RNase III-like enzyme Dicer into small interfering RNAs (siRNA) – short stretches of dsRNA ~21 nucleotides long. Once incorporated in the multi-protein complex RISC (RNA-induced silencing complex), these siRNAs guide post-transcriptional gene silencing through degradation of their target mRNA (RNA interference) or by inducing translational inhibition by the microRNA (miRNA) pathway (Figure 1) [1]. It is well established that RNA silencing functions as an adaptive antiviral immune response in plants and insects [2–4].

The importance of RNA interference (RNAi) during viral infection in mammals is still unclear. Mammalian cells have strong, non-specific responses to viral dsRNA. dsRNA interacts directly with cellular proteins, such as protein kinase R, retinoic acid-inducible gene I (RIG-1) or Toll-like receptor (TLR) 3 [5,6], which triggers signaling pathways that lead to the expression of type I interferons and the activation of non-specific RNases. In turn, type I interferons induce the expression of a large number of genes that create an antiviral state in the host cell. In principle, the evolution of these potent dsRNA-activated antiviral pathways in mammals might have superseded an antiviral function of the RNAi response. However, recent evidence reveals a major role for the RNAi machinery in the interplay between viruses and their mammalian hosts. In this review we discuss these studies and the advances towards the therapeutic exploitation of the RNAi pathway in the treatment of viral infection.

Lessons from plants and invertebrates: RNA silencing as an antiviral defense mechanism

RNA silencing in the context of viral infections has been studied extensively in plants, and many of the seminal insights into the antiviral role of RNAi stem from the analysis of plant–virus interactions. RNA silencing provides an adaptive, nucleic acid-based defense against DNA and RNA viruses that can induce systemic immunity in response to local infections, although viruses have evolved strategies to suppress the RNA-silencing pathway as a counter-defense (Box 1). An extensive analysis of this literature is available in several reviews [3,4,7].

RNA silencing also provides a natural antiviral defense in insects. Flock house virus (FHV), a member of the *Nodaviridae* family, which was isolated from naturally infected grass grubs (*Costelytra zealandica*), is targeted by the RNAi machinery in *Drosophila* S2 cells [8]. Accordingly, virus-derived siRNAs are produced during the infection, and depletion of RISC components results in an increase in viral RNA levels. The FHV B2 protein acts as a suppressor of RNAi and is essential for accumulation of viral RNA in the presence of a functional RNAi response [8].

Furthermore, several studies have established that RNAi protects insects from viral infection. Introduction of dengue virus genomic sequences with a Sindbis virus vector into cells of the mosquito *Aedes albopictus* resulted in RNAi-based resistance to subsequent challenges with dengue virus [2]. Similarly, a Semliki Forest virus replicon expressing sequences from the tick-borne hazara virus (HAZV) inhibited replication of HAZV in a cell line from the tick *Ixodes scapularis* [9]. Consistent with the idea that protection is mediated by RNAi, virus-derived siRNAs in the range of 21–23 nucleotides were detected in Sindbis virus infections of the silk moth *Bombix mori* and of the mosquito *Aedes aegyptii* [2,10]. An increase in titer and spread of O'nyong–nyong virus after depletion of two Argonaute proteins (Ago 2 and 3) – components of RISC – in the mosquito *Anopheles gambia* further supports an antiviral role of RNAi in suppression of alphavirus replication in its natural vector [11].

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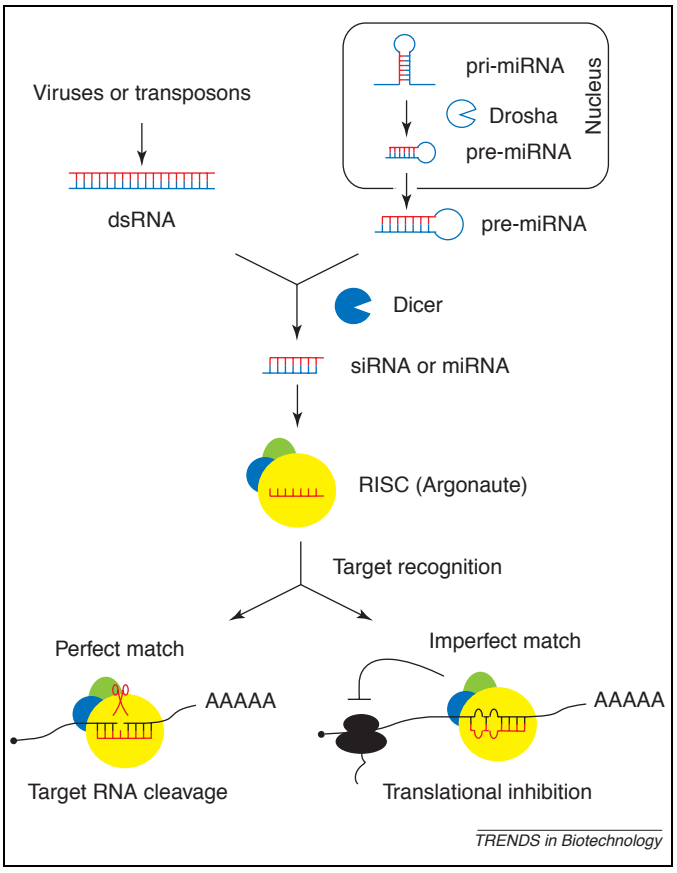

Figure 1. RNA silencing pathways. The double-stranded RNA silencing pathway is involved in two different modes of post-transcriptional gene silencing [1,58]. In the 'cleavage mode' of RNAi, double-stranded RNA precursors are cleaved by Dicer into short interfering RNAs. Once incorporated in RISC, these siRNAs will guide cleavage of their target RNA upon recognition of a complete complementary sequence. Argonaute 2 is the catalytic engine of RISC in mammals, responsible for cleavage of the target RNA by its RNase H-like domain. The 'translational repression mode' of RNA silencing is initiated by microRNA genes, a class of small, non-coding RNAs encoded in the genome [58]. Primary miRNA transcripts (pri-miRNAs), ranging from several hundred to several thousand base pairs, are cleaved by the nuclear RNase III-like enzyme, Drosha, into ~70 nucleotide stem–loop structures, the precursor miRNAs (pre-miRNA). These pre-miRNAs are exported to the cytoplasm by the nuclear export factor, Exportin 5, where they are cleaved into their mature ~22 nucleotide form by Dicer. Mature miRNA are loaded into a RISC-like complex, which guides inhibition of translation without destroying the target mRNAs. Although the precise mechanism of translational inhibition is unknown, it seems to be independent of Argonaute 2. In general, miRNAs exhibit only near-complete complementarity to their target site. However, some miRNAs in mammals exhibit complete complementarity to their target mRNA, directing mRNA cleavage. In addition to these post-transcriptional modes of gene silencing, the RNAi machinery has been implicated in transcriptional gene silencing, *de novo* methylation of DNA and changes in chromatin structure in several organisms [59]. Figure adapted with permission [60].

Although viruses that naturally infect the nematode *Caenorhabditis elegans* have not been identified, the antiviral function of RNAi has been established using two viruses with a broad host-range: FHV and vesicular stomatitis virus (VSV). Accordingly, virus-derived siRNAs are produced during replication, and enhanced virus replication was observed in nematode mutants that are defective in RNAi [12,13].

Viral infections and the RNAi response in mammals

Does RNAi contribute to the antiviral defense mechanism in mammalian cells? Mammals have evolved highly sophisticated and effective systems of innate and adaptive immune responses to infections, based on protein recognition; therefore, they might not need the more ancient nucleic acid-based response. Furthermore, as discussed above, dsRNA activates non-specific RNA degradation and a generalized repression of protein synthesis, primarily mediated by protein kinase R (PKR) and RNase L [5]. It might, thus, be argued that mammalian cells do not need an RNAi-based antiviral response, that the principal role of RNAi in mammals is regulation of endogenous gene expression and that the antiviral role of RNAi in plants and insects stems from a functional diversification of the proteins of the RNAi pathway. For example, mammals only encode a single Dicer gene, whereas *Arabidopsis thaliana* encodes four different Dicer-like (DCL) genes. These different DCLs might enable the plant to recognize different types of double-stranded RNA. Indeed, a loss of function of DCL2 resulted in a reduction in siRNA production in only one out of three viruses tested [14], supporting the notion that different DCLs might have specialized functions. Furthermore, the RNAi defense against certain viruses relies on the systemic spread of dsRNA in plants (Box 1). By contrast, RNAi in mammals is thought to be a cell-autonomous process because of the apparent lack of mechanisms for the amplification and spread of the silencing signal [15]. However, as discussed below, several intriguing studies show that mammalian viruses do interact with the cellular RNAi machinery. For example, several viruses encode modulators of the mammalian RNAi response, suggesting that RNAi might indeed regulate viral replication. In addition, it is now well accepted that RNAi can be used, therapeutically, to target and control viral infection in mammals.

Antiviral activity of the RNAi response: suppressors of RNAi and virus-derived siRNAs

Identification of suppressors of the RNAi machinery in animal viruses suggests that these viruses are naturally targeted by RNAi. Two interferon antagonists, the dsRNA-binding proteins NS1 from influenza virus and the E3L protein from vaccinia virus, might substitute the function of the FHV-silencing suppressor, B2, in *Drosophila* S2 cells [16]. Furthermore, influenza NS1 binds siRNAs *in vitro*, and suppresses RNA silencing in plants [17,18]. The B2 protein from nodamura virus (the only member of the *Nodaviridae* family that naturally infects mammals) can inhibit short hairpin RNA (shRNA) and siRNA-initiated RNAi in mammalian cells. B2 can bind pre-Dicer substrates, inhibiting Dicer processing [19].

Thus, RNAi-suppressive activity, encoded by viruses that infect mammalian cells, suggests that these viruses are targets of the RNAi machinery; however, the suppressive role of these factors was examined by overexpression of the suppressor and not in the context of infection. Thus, the physiological role of these silencing suppressors in the setting of viral infections awaits further investigation. For example, the absence of these suppressors should render these viruses hyper-sensitive to RNAi. Influenza replicates poorly in the absence of NS1 in mammalian cells, underlying an important role for NS1 in the establishment of productive infection. However, the defect in virus replication is absent in type I interferon-deficient cells

Box 1. Lessons from plants: RNA silencing as an antiviral defense

Several lines of evidence support an antiviral role for the RNA-silencing machinery in plants.

i) Virus-derived siRNAs are produced during infection by DNA and RNA viruses [61]. Double-stranded RNA is produced during virus replication and is processed by one of the four plant Dicer-like genes into siRNAs. Sources of dsRNA in RNA virus infections might include the dsRNA intermediate in replication or local basepaired structures in ssRNA viruses [62]. Overlapping transcripts derived from bidirectional transcription might be the source for dsRNA during replication by geminiviruses, viruses with a ssDNA genome [63].

ii) Plants in which components of the RNA silencing pathway are functionally impaired support increased virus replication and spread. For example, *Arabidopsis* mutants, in which Dicer-like 2 or the RNA-dependent RNA polymerase 6 (RDR6 or SDE1/SGS2) are functionally inactivated, display increased viral titers and more severe disease symptoms after infection with certain viruses [14,64].

iii) A local and systemic silencing signal is initiated upon virus infection. A mobile silencing signal can travel ahead of the viral infection to uninfected tissues, thereby limiting viral infection when the virus reaches these cells [3]. Amplification and systemic spread of the silencing signal is dependent on the combined action of the host-encoded RNA-dependent RNA polymerase RDR6 and the putative helicase SDE3 [65].

iiii) Viruses have evolved suppressors of the RNA silencing pathway as a counter-defense against this potent antiviral pathway [4,7]. Although the mechanism of many suppressors of RNA silencing await elucidation, it is clear that different aspects of the silencing machinery can be targeted by these proteins. For example, tombusvirus p19 binds siRNAs with high specificity, thus sequestering siRNAs and preventing the incorporation in RISC [66], whereas the cucumber mosaic virus 2b protein suppresses the systemic spread of the silencing signal [67]. In addition to these RNA viruses, silencing suppressors have been identified in DNA viruses, further supporting the notion that DNA viruses can be targeted by the RNAi machinery as well.

[20], suggesting that the major role of NS1 is the evasion of the interferon response rather than suppressing an antiviral RNAi response. Intriguingly, defects in RNA replication of nodamura virus mutants without functional B2 protein varied from negligible to severe in different mammalian cell lines [21]. The basis for these differences has not been established.

Inhibition of the RNAi pathway, mediated by two non-coding viral RNAs, was observed for adenovirus, a virus with a dsDNA genome. The ~160 nucleotide adenoviral virus-associated (VA) RNA I and II are expressed at high levels during infection and are involved in blocking activation of protein kinase R. In addition, VA RNAs inhibit RNAi initiated by shRNAs and miRNAs but not by siRNAs. In this case, inhibition was mediated by competitive binding to the nuclear export factor Exportin 5 and to Dicer [22,23]. The VA RNAs are substrates for Dicer, and siRNAs derived from the terminal stem of VA I and II accumulate in infected cells and are incorporated into RISC [23]. It is, thus far, unclear how the virus benefits from these interactions. The VA RNA genes do not overlap with protein-coding genes, and VA-siRNA-loaded RISC will, therefore, not affect virus-specific mRNAs. A possibility might be that the VA RNAs saturate the RNAi machinery and thus prevent Dicer and RISC from targeting the coding sequences. An alternative possibility is that the virus actually exploits the RNAi machinery and that the VA-siRNAs act as miRNAs to inhibit host gene expression.

The presence of virus-derived siRNAs provides direct proof that viral sequences are cleaved by Dicer. Thus far, identification of virus-derived siRNAs has been unsuccessful in tissue culture models for infection with hepatitis C virus (HCV), HIV and yellow fever virus [24]. Recently, however, a specific virus-derived siRNA (v-siRNA) was identified in HIV-1-infected cells [25]. Thus far, there is no explanation for the disparity between the identification of a v-siRNA and the earlier inability to clone HIV-specific siRNAs [24], although this might be because of the use of different strains of virus or a low abundance of the v-siRNA. The precursor for this v-siRNA appears to be derived from an inverted repeat in the viral genome [25]. This inverted repeat can generate a 21-nucloetide double-stranded structure (using intermolecular base-pairing) or a hairpin structure with a 19-base-pair stem and a 200-base-pair nucleotide loop (using intramolecular base-pairing), both flanked by long stretches of single-stranded siRNA. Given the preference of Dicer to cleave progressively from the termini of dsRNA molecules [26], it is not obvious how Dicer can process such structures efficiently.

Furthermore, the HIV-encoded tat protein was shown to inhibit Dicer activity *in vitro* [25]. The authors speculate that the suppressor function of tat is essential for efficient replication in the presence of Dicer activity targeting the viral RNA. However, it is not clear why the virus relies on the action of a suppressor of RNAi rather than mutating the target sequence. The high conservation in this region among different isolates suggests that this duplex has been selected during evolution but the precise role in virus replication is still unclear. Nevertheless, the presence of RNAi suppressor activity in RNA viruses, and the identification of virus-derived siRNA in infected cells, supports the notion that the RNAi machinery might possess antiviral activity. However, the functional relevance of these interactions for viral pathogenesis awaits further investigation.

Endogenous miRNAs interacting with viruses

Recently, it was described that endogenous microRNAs (miRNAs) might provide antiviral defense against the retrovirus primate foamy virus (PFV) in human cells [27]. A potential target site for miRNA 32 (miR-32) was identified in the genome of PFV: this region mediates translational inhibition of PFV transcripts. Inhibition of miR-32 function by an antisense oligonucleotide or by mutagenesis of the miR-32 target site resulted in an increase in replication. To counter an antiviral effect of miR-32, the virus encodes the tas protein, which suppresses miRNA-mediated translational inhibition and target-RNA cleavage. It is surprising that the virus would depend on the RNAi suppressor function of tas to escape the antiviral effect of miR-32 rather than mutating the miRNA target sequence, particularly because the authors show that a mutant with a mutated target site is viable.

The first eight 5′ nucleotides of a miRNA are the main determinant of specificity for target recognition and are sufficient for translational inhibition by miRNAs. The authors, therefore, hypothesize that many endogenous

miRNAs might have direct antiviral effects through fortuitous recognition of foreign nucleic acids in addition to their normal cellular function. Thus, by analogy with the protein-based immune system, an antiviral response, initiated by Dicer cleavage of viral dsRNA, might represent the adaptive branch of the nucleic acid-based immune response, whereas the endogenous antiviral function of miRNAs might represent the innate branch of this response.

An intriguing interaction between a host miRNA and a viral genome has been described for HCV [28]. A target site for miRNA 122 is present in the 5′ UTR of HCV and this miRNA is highly expressed in the liver – the natural target for HCV; however, by sharp contrast to what might be expected, this interaction seems to be beneficial for the virus: inhibition of miRNA function or mutagenesis of the target site inhibits viral replication. The reasons for this phenomenon are currently unknown.

Viruses using the RNAi machinery: virus-encoded miRNAs

Given the enormous potential of miRNAs to modulate gene expression, it is not surprising that viruses have exploited the RNA-silencing pathway to their advantage. Initially, virus-encoded miRNAs were identified while cloning the small RNA profile in cells infected with Epstein–Bar virus (EBV) [29]. These miRNAs are encoded as fold-back precursors in the untranslated regions and introns of two viral genes. Further virus-encoded miRNAs have been identified in several additional DNA viruses, including Kaposi's sarcoma-associated herpesvirus (KSHV) and cytomegalovirus (CMV), by computer predictions and experimental verification [30]. Although targets for the EBV miRNAs have been predicted [29], the functional significance of a viral miRNA has only been established in the case of SV40. This viral miRNA is expressed from the late transcript and is complementary to the early viral transcript. The viral miRNA targets the early transcript for degradation, reducing the expression of small and large T antigens without a concomitant reduction in viral titer. Infected cells were less susceptible to lysis by T-antigen-specific cytotoxic T cells, suggesting that SV40 exploits the RNAi machinery to regulate viral gene expression, thereby reducing its susceptibility to the adaptive immune response [31].

RNAi as an antiviral therapeutic approach

The exploitation of the RNAi pathway might enable the development of effective antiviral therapies. Expression of endogenous genes can be experimentally suppressed by delivery of intermediates in the RNAi pathway. This can be accomplished in mammalian cells by delivery of synthetic siRNAs or by plasmid-driven expression of short hairpin (sh)RNAs, similar in structure to pre-miRNAs [32]. Pretreatment of cells with virus-specific siRNAs programs RISC for destruction of viral RNA. Accordingly, the replication of a large number of viruses can be inhibited efficiently by pre-treatment of tissue culture cells with synthetic siRNA or with plasmids expressing shRNAs (Table 1). Replication of several viruses, including many important human pathogens, with different genetic make-ups and replication strategies has been inhibited by RNAi. Indeed, it appears that, in principle, all viruses can be targeted by RNAi, provided that efficient siRNAs are designed. Although genomic and replicating viral RNA are usually protected by proteinaceous or membranous structures, some single-stranded viral RNAs (such as translating RNAs) are sensitive to the RNAi machinery. Indeed, only mRNA has been identified

Table 1. RNAi-mediated suppression of mammalian virus replication in tissue culture

Virus family	Genome*	Virus	si/shRNA	Refs
RNA viruses				
Coronaviridae	(+) ssRNA	SARS-associated CoV	siRNA	[68]
Flaviviridae	(+) ssRNA	Hepatitis C virus	siRNA; shRNA	[55,56,69]
		West Nile virus	shRNA	[70]
Picornaviridae	(+) ssRNA	Coxsackievirus B3	siRNA	[38]
		Foot-and-mouth disease virus	shRNA	[71]
		Hepatitis A virus	siRNA	[72]
		Human rhinovirus 16	siRNA	[73]
		Poliovirus	siRNA	[51]
Paramyxoviridae	(-) ssRNA	Respiratory syncytial virus	siRNA	[35]
		Human parainfluenza virus-3	siRNA	[74]
Rhabdoviridae	(-) ssRNA	Vesicular stomatitis virus	siRNA	[74]
Orthomyxoviridae	Segmented; (-) ssRNA	Influenza A virus	siRNA; shRNA	[33,70]
Deltavirus	Circular; (-) ssRNA	Hepatitis delta virus	siRNA	[34]
Reoviridae	Segmented; dsRNA	Rotavirus	siRNA	[75]
Reverse-transcribing RNA and DNA viruses				
Retroviridae	ssRNA	Human immune deficiency virus-1	siRNA; shRNA	[57]
Hepadnaviridae	Circular; partially dsDNA	Hepatitis B virus	siRNA; shRNA	[76]
DNA viruses				
Herpesviridae	dsDNA	Herpes simplex virus-1 (HHV1)	siRNA	[77]
		Human cytomegalovirus (HHV5)	siRNA	[78]
		Epstein Bar virus (HHV4)	shRNA	[79]
		Human herpes virus 6B	siRNA	[80]
		Murine herpesvirus 68	SiRNA	[81]
Papovaviridae	Circular; dsDNA	Human papiloma virus 18	SiRNA	[82]
		JC virus	SiRNA	[83]

*(+), positive stranded; (-) negative stranded; ss, single stranded; ds, double stranded.

Table 2. Use of RNAi in *in vivo* animal models of virus infections

Virus family	Virus	shRNA/siRNA formulation	Method of delivery	Main outcome	Refs
Coronaviridae	SARS-associated CoV*	siRNA in D-glucose/water carrier	Intratracheal administration	Reduced viral RNA and lung pathology	[84]
Flaviviridae	West Nile virus	siRNA	Hydrodynamic injection	Reduced viral load, partial protection from lethal infection	[85]
Picornaviridae	Foot-and-mouth disease virus	shRNA plasmid	Subcutaneous injection in neck	Protection from lethal infection	[71]
Paramyxoviridae	Respiratory syncytial virus	Naked siRNA or siRNA complexed with lipofection agent	Intranasal delivery	Reduced lung titers and pulmonary pathology	[41]
	Respiratory syncytial virus	shRNA plasmid complexed with chitosan nanoparticle	Intranasal delivery	Reduced lung titers and pulmonary pathology	[40]
	Parainfluenza virus	siRNA complexed to lipofection agent	Intranasal delivery	Reduced lung titers	[41]
Orthomyxoviridae	Influenza A virus	siRNA or shRNA plasmid complexed with cationic polymer polyethyleneimine	Intravenous injection	Reduced lung titers	[39]
	Influenza A virus	Naked siRNA and siRNA complexed to lipofection agent	Combination of hydrodynamic injection and intranasal delivery	Reduced lung titers and protection from lethal infection	[86]
Hepadnaviridae	Hepatitis B virus	shRNA plasmid	Hydrodynamic injection	Reduced virus replication and viral antigen	[46]
	Hepatitis B virus	siRNA	Hydrodynamic injection	Reduced virus replication and viral antigen	[47]
	Hepatitis B virus	shRNA expressed by recombinant Adenovirus	Intravenous injection	Reduced virus replication and viral antigen, clearance of viral RNA in ongoing infection	[42]
	Hepatitis B virus	Chemically modified siRNA in liposome	Intravenous injection	Reduced virus replication and viral antigen	[49]

*This study was performed in Rhesus macaques (*Macaca mulatta*) all other studies were performed in small rodent models.

as the target for RNAi during replication of the negative-strand RNA viruses – influenza A, hepatitis delta virus (HDV) and respiratory syncytial virus (RSV) [33–35] – and the double-stranded rotavirus [36]. In these cases, the genomic RNA and the complementary RNA might be shielded from cleavage by RISC by virtue of either their nuclear localization (influenza, HDV), their replication in non-membrane-bound inclusions (viroplasms, rotavirus) or their association with nucleocapsid protein (RSV). In positive-stranded picornaviruses, in which the viral genomic RNA is used both as a messenger for translation and as a template for negative-strand synthesis, only the positive RNA strand is targeted by RNAi [37,38].

Several groups have successfully extended these tissue culture observations to *in vivo* animal models of virus infections (Table 2). In general, the most effective suppression of virus replication in these studies was observed when the animals were pre-treated with siRNAs before a viral challenge. However, an antiviral effect of RNAi was also observed when siRNAs were administered between two and three days after infection with influenza and RSV [39–41]. Importantly, in hepatitis B virus (HBV)-transgenic mice, in which HBV replication is ongoing, virus replication might be suppressed to undetectable levels up to 26 days after delivery of a virus-specific shRNA by a recombinant adenovirus [42]. Although clinical applications are perhaps several years away, these studies provide proof-of-principle of a novel class of RNAi-based prophylactic and therapeutic strategies against acute and chronic virus infections.

Obstacles to antiviral RNAi therapy: delivery

Delivery of siRNA to the sites of virus replication and then into cells is one of the major obstacles preventing clinical applications. RNAi can be induced by synthetic siRNAs or by plasmid-based expression of shRNA in the cell [32]. The latter, which can be expressed from viral vectors, has the benefit of prolonged expression of shRNA and might be useful in the teatment of chronic infections. Indeed, an adenovirus expressing shRNA was successfully used to suppress established HBV replication for a prolonged period [42]. However, the use of these vectors might encounter similar problems and limitations as gene therapy, including issues relating to safety, toxicity, immunogenicity and delivery [43]. Synthetic siRNAs can be delivered as a complex with a carrier or as naked nucleic acid. The introduction of chemical modifications, such as partial phosphorothioate backbone and 2′-*O*-methyl sugar modifications, results in enhanced serum stability and might improve the pharmacokinetic properties of siRNAs [44]. Delivery vehicles can enhance the delivery of siRNA to specific organs after intravenous injection. For example, cholesterol-conjugated siRNA accumulated in several organs and induced silencing of the endogenous apolipoprotein B gene in the liver [44], whereas polyethylenimine facilitates delivery of siRNAs to the lung, where inhibition of influenza A virus was achieved [39].

Viruses differ greatly with respect to cell and tissue tropism, which will pose specific hurdles to the delivery issue. For example, chronic infections will require prolonged expression or repeated delivery of siRNAs. Local delivery of siRNAs might be feasible for the treatment of acute viruses with a relatively restricted tissue tropism, such as RSV, influenza and parainfluenza virus, and other respiratory viruses. Indeed, intranasal delivery was a successful approach to deliver siRNA complexed to either lipid carriers, or to the natural

polymer chitosan, to the lungs [41,45]. Use of lipids will increase drug costs, increase toxicity and complicate regulatory approval. In that respect, it is encouraging to note that naked siRNAs significantly suppressed RSV virus titers in the lung, albeit with lower efficiency than the lipid-complexed siRNA [41]. High-volume hydrodynamic injection has been used for delivery to the liver for the treatment of hepatitis viruses [46,47]. Local vein injection for delivery of siRNAs is also an alternative, given the recent finding that low-volume renal vein injection of a siRNA targeting *Fas* was as effective as high-volume hydrodynamic injection in a renal ischemia–reperfusion disease model [48]. Alternatively, cholesterol conjugation [44] or lipid-encapsulation might provide a way to deliver siRNAs, intravenously, for treatment of viral hepatitis [49]. For the treatment of HIV-1 infection – with its tropism for T cells, monocytes and macrophages – *ex vivo* transduction of haematopoetic stem cells with lentiviral vectors expressing anti-HIV siRNAs, thus generating an HIV-1 resistant pool of cells, has been proposed recently, and this approach might soon be tested in clinical trials [32].

Finally, an attractive delivery vehicle for cell type-specific targeting was recently described, based on a fusion protein comprising an antibody Fab fragment coupled to the nucleic acid-binding protein protamin [50]. Using the Fab fragment of an HIV-1 envelope-specific antibody, delivery of HIV-1-specific siRNAs to primary T cells resulted in inhibition of HIV-1 production. Although the feasibility of this approach in the setting of viral infections need to be established *in vivo*, the authors demonstrate that siRNAs might be delivered to HIV-envelope-expressing tumor cells *in vivo*.

Obstacles to antiviral RNAi therapy: viral escape

Viruses are notorious for their ability to develop resistance to antiviral drugs; in this respect, it bears little surprise that viral escape from RNAi suppression has been described. Single mismatches within the targeted region, or even its entire deletion, resulted in escape from RNAi by poliovirus, HCV and HIV-1 [37,51–54]. Initiating the RNAi response using long dsRNA (in cells that lack an interferon response) or using a pool of siRNAs by *in vitro* enzymatic cleavage of a 1000 bp dsRNA fragment delayed viral escape of poliovirus [37]. Although these approaches generate a complex mixture of different siRNAs, the use of two defined siRNAs was sufficient to delay escape of a HCV replicon [54]. Treatment with a combination of different siRNAs, providing an RNAi equivalent of a multi-drug regimen, might, thus, be a feasible approach to prevent viral escape. The advantage of RNAi as an antiviral therapy compared with other types of antiviral drugs is that the design of multiple siRNAs is relatively straightforward. An alternative approach to delay viral escape might be based on the choice of target site. The untranslated regions (UTRs) of RNA viruses contain complex RNA structures that provide binding sites for cellular and viral proteins and sites for RNA–RNA interactions that are often essential for virus replication. Targeting these regions by RNAi might prevent viral escape because point mutations in these structures might lead to loss of function. By contrast, silent mutations in the coding region might not compromise viral fitness and readily enable the virus to escape. Although 3′ UTRs of cellular mRNA are routinely used as targets for RNAi, it is important to determine the susceptibility of viral untranslated regions because interaction in the UTR with proteins or RNA might shield them from RISC-mediated recognition and cleavage. Indeed, it has been observed that certain regions in the HCV 5′ UTR can be targeted by RNAi, whereas other regions seem to be relatively resistant [55,56]. Finally, targeting the host factors required for virus replication, such as the virus receptor, is perhaps an effective way to prevent virus escape. This approach has been employed, effectively, in several studies [57].

Conclusion

Although it is established that RNAi serves as an adaptive nucleic acid-based immune response to viruses in plants and insects, evidence is emerging to support the concept that the RNAi machinery naturally interacts with mammalian viruses. These interactions include the exploitation of the miRNA pathway by DNA viruses and the innate nucleic acid-based antiviral response of endogenous miRNAs. In addition, the presence of RNAi suppressor activity and virus-derived siRNAs in selected viruses imply that RNAi also has a role in the control of virus replication. The antiviral RNAi pathway can be exploited, experimentally, to suppress virus replication, *in vitro* and in animal models, providing proof-of-principle for a novel class of antiviral drugs based on RNAi technology.

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