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RNA Interference: Its Use as Antiviral Therapy

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Abstract RNA interference (RNAi) is a sequence-specific gene-silencing mechanism that has been proposed to function as a defence mechanism of eukaryotic cells against viruses and transposons. RNAi was first observed in plants in the form of a mysterious immune response to viral pathogens. But RNAi is more than just a response to exogenous genetic material. Small RNAs termed microRNA (miRNA) regulate cellular gene expression programs to control diverse steps in cell development and physiology. The discovery that exogenously delivered short interfering RNA (siRNA) can trigger RNAi in mammalian cells has made it

into a powerful technique for generating genetic knock-outs. It also raises the possibility to use RNAi technology as a therapeutic tool against pathogenic viruses. Indeed, inhibition of virus replication has been reported for several human pathogens including human immunodeficiency virus, the hepatitis B and C viruses and influenza virus. We reviewed the field of antiviral RNAi research in 2003 (Haasnoot et al. 2003), but many new studies have recently been published. In this review, we present a complete listing of all antiviral strategies published up to and including December 2004. The latest developments in the RNAi field and their antiviral application are described.

Keywords RNA interference · siRNA · shRNA · RISC · Virus · Antiviral therapy

1 Introduction

RNA interference (RNAi) is certainly not the first mechanism with therapeutic potential that is based on nucleic acid. The idea of using nucleic acid-based inhibitors of gene expression stems from research performed in the 1980s when antisense DNA oligonucleotides could be chemically synthesized for the first time. Scientists had high hopes for antisense, but thus far only a single drug, called Vitravene, cleared the Food and Drug Administration (FDA) in 1998 and is currently used to treat cytomegalovirus-induced eye infections in acquired immunodeficiency syndrome (AIDS) patients. The exploitation of DNA-based therapeutic agents remains limited because of delivery problems and unpredictable effectiveness. Similarly, ribozymes once held promise, but not one ribozyme-based drug has come to the market. RNAi is about to change the momentum. Acuity Pharmaceuticals in Philadelphia filed an investigational new drug application with the FDA in August 2003 to begin testing small interfering RNA (siRNA) treatment for the wet form of age-related macular degeneration (AMD). Still, it could be years before the first RNAi drug is approved for consumer use; but the field is moving at an unprecedented speed. The RNAi pathway is commonly considered a cellular defence mechanism against viruses, and it can be used as a therapeutic tool against pathogenic viruses. We will summarize the current status of RNAi-mediated inhibition of virus replication and discuss the possibilities for the development of RNAi-based antiviral therapeutics.

2 The Biology of RNA Interference

RNAi is a cellular regulatory pathway triggered in response to double-stranded RNA (dsRNA) (Hannon 2002). Since its first description in *Caenorhabditis elegans*, RNAi has been found to exist in many eukaryotic organisms and to be involved in an extraordinary number of gene-silencing phenomena (Fire et al.

1998; Hannon 2002). The RNAi machinery consists of a conserved core of factors with roles in recognizing, processing and effecting the responses to dsRNA. MicroRNAs (miRNAs) are a growing family of small non-protein-coding regulatory transcripts found in many eukaryotic organisms. miRNAs are processed via the RNAi machinery, and some have been shown to regulate the expression of homologous target-gene transcripts. miRNAs were first described in *C. elegans*; the lin-4 and let-7 transcripts. Both target the 3′ untranslated regions (3′-UTRs) of developmental transcription factors and suppress their translation (Grishok et al. 2001; Reinhart et al. 2000; Vella et al. 2004). Current estimates for the number of miRNAs represent 1% of the predicted number of genes in a mammalian genome, similar to the proportion represented by large gene families such as transcription factors. Computational methods are being used to identify targets with the expectation that the results will provide clues as to the mechanism of action of a particular miRNA (Lai 2004). As more miRNA:target pairs were described, it became apparent that regulation of development might be a common theme in miRNA biology. miRNAs and their targets have been found to affect diverse processes, including flowering time and leaf patterning in *Arabidopsis*, neuronal asymmetry in *C. elegans*, and developmentally regulated cell proliferation in *Drosophila* (Aukerman and Sakai 2003; Brennecke et al. 2003; Kidner and Martienssen 2004; Palatnik et al. 2003). Little is known about the roles of miRNAs in mammals, but the field is moving fast (Miska et al. 2004).

2.1 miRNA Biosynthesis and DICER-Mediated Processing

miRNA genes are often located in clusters that appear to be transcribed as a polycistronic transcript (Lee et al. 2002b). Perhaps surprisingly, neither the miRNA promoters nor the RNA polymerase responsible for miRNA transcription has been characterized in detail thus far. However, recent evidence indicates that human miRNAs are processed from capped, polyadenylated transcripts that can also function as messenger (m)RNAs (Cai et al. 2004). It is also clear that miRNA genes are often under strict developmental- and tissue-specific control (Dostie et al. 2003; Houbaviy et al. 2003; Krichevsky et al. 2003; Lagos-Quintana et al. 2002; Moss and Tang 2003). Many primary miRNA transcripts are predicted by computer algorithms to adopt elaborate stem-loop structures (Lau et al. 2001; Lee and Ambros 2001; Mourelatos et al. 2002). Cleavage of the stem-loops by the RNase III enzyme Drosha liberates approximately 70-nucleotide (nt) precursor miRNAs (pre-miRNAs) (Lee et al. 2003b). These RNAs have the characteristic two-nt 3′ overhang end structure left by the staggered cut of RNase III enzymes. Recent studies have shown that Exportin-5 mediates the nuclear export of pre-miRNAs in a Ran–guanosine triphosphate (GTP)-dependent manner (Bohnsack et al. 2004; Lund et al. 2004; Yi et al. 2003). Interestingly, Exportin-5-mediated nuclear export of another

cargo RNA, the adenovirus VA1 noncoding RNA, also requires a two- to three-nt 3′ overhang (Gwizdek et al. 2004), suggesting that the structural determinants of Exportin-5 recognition may be implicit in Drosha processing. Entry into the cytoplasm brings pre-miRNAs into contact with Dicer, a predominantly cytoplasmic enzyme (Billy et al. 2001; Findley et al. 2003; Provost et al. 2002). Dicer cleaves pre-miRNAs into mature ~22mer miRNAs, but it can also cleave dsRNA into ~22mer siRNAs (Bernstein et al. 2001; Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Knight and Bass 2001). Dicer is a modular enzyme composed of two RNaseIII domains, a DExH/DEAH box RNA helicase domain and a PAZ domain, as well as a domain of unknown function (DUF283) and a double-stranded RNA-binding motif (Bernstein et al. 2001).

2.2 RISC-Mediated Target RNA Cleavage

Dicer cleavage is followed by release of the mature miRNA or siRNA, and its incorporation into a RISC (RNA-induced silencing complex) effector complex whose diverse functions include mRNA cleavage, translational suppression, transcriptional silencing and heterochromatin formation. The *C. elegans* double-stranded RNA-binding protein RDE-4 and its *Drosophila* homologue R2D2 facilitate the transfer of siRNAs to RISC (Liu et al. 2003; Tabara et al. 2002). There is also likely to be a number of additional components that aid in RISC assembly (Tomari et al. 2004a), particularly those that unwind siRNAs or miRNAs and through this action determine which RNA strand is chosen for silencing (Schwarz et al. 2003). Which strand of the miRNA or siRNA duplex is preferentially incorporated into RISC is also determined by the thermodynamic properties of the nucleic acid duplex (Martinez et al. 2002a; Schwarz et al. 2003).

RISC ribonucleoprotein complexes contain members of the PAZ–Piwi domain Argonaute family of proteins, siRNAs or miRNAs, and miRNA/siRNA complementary mRNAs. The Argonaute protein recognizes the 3′ overhang of the single-stranded (ss)RNA and is a crucial component of RISC. The loaded ssRNA guides the search for mRNA with complementary sequences and defines the actual site of cleavage (~10 bases from the 3′ overhang). The first crystal structure of an archaeal Argonaute protein revealed an RNaseH-like fold with a catalytic Asp-Asp-Glu (DDE) motif for RNA cleavage, suggesting that this protein is the actual RNA slicer (Parker et al. 2004; Song et al. 2004). Mammalian cells have four Argonaute homologues, but recent evidence suggests that Ago2 has the RNA-cleaving activity (Liu et al. 2004a; Rand et al. 2004). There may be a division of labour between Argonautes. For instance, *Drosophila melanogaster* was recently shown to have Ago2 for unwinding of siRNA, which is a prerequisite for siRNA-mediated cleavage, and Ago1 interacts directly with Dicer, leading to stabilization of mature miRNAs (Okamura et al. 2004). The endonucleolytic cleavage by RISC generates two RNA frag-

ments with a 3′ hydroxyl and 5′ phosphate (Schwarz et al. 2004). These mRNA fragments must be eliminated because they may encode unwanted polypeptide fragments when translated. The fate of the mRNA cleavage products remained unclear for some time, but recent evidence has implicated a 5′ to 3′ exonuclease, the cytoplasmic AtXRN4 enzyme in *Arabidopsis*, in removal of 3′ fragments (Gazzani et al. 2004; Souret et al. 2004).

RISC contains a number of accessory factors, some of which have activities necessary for effector function. The precise biochemical mechanisms whereby RISCs carry out their functions are unknown. Two well-characterized RISC activities are mRNA cleavage and translational suppression. RISC is a multiple-turnover enzyme complex, and, once incorporated, an siRNA or miRNA can direct multiple rounds of target cleavage (Hutvagner and Zamore 2002). Whether RISC can also act in a catalytic manner in mediating translational suppression is not clear. It is also not known whether miRNA–mRNA duplexes require specific features (e.g. a mismatch) to be recognized by factors that mediate the translational repression. Recent evidence indicates that the primary function of the miRNAs is to guide their associated proteins to the mRNA. Tethering Ago proteins to the 3′-UTR of an mRNA by other means also resulted in translational repression (Pillai et al. 2004).

2.3
RNAi and the Impact on Chromosome Structure and Transcription

Although miRNA molecules are thought to act at the post-transcriptional level by interfering with mRNAs, there is accumulating evidence that some of them, and other non-coding RNA molecules, are involved in transcriptional silencing and heterochromatin formation as well. RNAi is known to work transcriptionally in plants by methylating gene promoters with sequences complementing the RNAs. De novo cytosine methylation of genomic DNA was shown to occur in plants infected with RNA viroids whose sequences were homologous to the methylated genomic sequences (Wassenegger et al. 1994). This process is referred to as RNA-directed DNA methylation (RdDM). Subsequently, dsRNA targeting a promoter region was shown to induce RdDM and to trigger transcriptional silencing. This silencing was accompanied by siRNA production, pointing to an RNAi-like mechanism for gene silencing at the transcriptional level (Mette et al. 1999). There is recent evidence that DNA methylation and gene silencing can also be induced by siRNA in human cells (Kawasaki and Taira 2004; Morris et al. 2004). Cells typically pass this DNA-regulating modification on to daughter cells, possibly permitting more lasting inhibitory effects.

RNAi has been linked with heterochromatin formation, gene silencing and chromosome segregation in fission yeast. Transcripts derived from the outer centromere repeats of the yeast chromosomes are chopped up by the RNAi machinery, thus forming siRNAs that are required for the formation of het-

erochromatin over the outer repeats. Chromosome analysis in a mammalian cell with a conditional knockout of Dicer suggested that RNAi does also play a role in heterochromatin formation at centromeres in vertebrates (Fukagawa et al. 2004).

This second DNA pathway could provide an alternative means to inhibit certain viruses by RNAi, in particular viruses that utilize DNA transcription as part of their replication strategy, which includes all DNA viruses and retroviruses. Compared to the RNA phase, the existence of a DNA phase of the RNAi machinery could provide longer-lasting effects and thus open new therapeutic strategies. On the other hand, a broader mechanism of action does also increase the likelihood of unintended consequences through off-target silencing.

3 Optimizing the siRNA Inhibitor

The results of detailed biochemical studies suggest a need to revise the current design rules for the construction of siRNAs (Khvorova et al. 2003; Schwarz et al. 2003). One could select RNA targets for which the corresponding siRNA molecule has an optimal thermodynamic signature. Naturally occurring miRNAs show a strong bias for accumulating only one strand into functional RISC complexes (Bartel 2004). In the course of identifying more active and more specific siRNAs, it was noticed that the sequence composition of the siRNA duplex influences the ratio of the "sense" and "antisense" (complementary to the target gene) siRNAs entering the RISC complex (Khvorova et al. 2003; Schwarz et al. 2003). Particularly important is a low base-pairing stability at the 5′ end of the antisense strand relative to the 3′ end, which increases the chance that this antisense strand enters the RISC complex. An additional advantage of such biased uptake in RISC is that there will be less off-target effects through RISC complexes that are loaded with the sense strand. In *Drosophila*, the orientation of the Dicer-2/R2D2 protein heterodimer determines which siRNA strand associates with the core RISC protein Argonaute 2 (Tomari et al. 2004b). R2D2 binds the siRNA end with the greatest double-stranded character, thereby orienting the heterodimer on the siRNA duplex. Strong R2D2 binding requires a 5′-phosphate on the RNAi strand that is subsequently excluded from RISC. This explains why relatively low base-pairing at the 5′ end of the antisense strand is beneficial.

In case the selected siRNA is still sub-optimal, one could improve its thermodynamic signature by altering the structure of the siRNA duplex. This can be done by introduction of weak G-U base-pairs, internal loops or bulges at distinct positions within the duplex, obviously without changing the antisense strand that mediates subsequent mRNA-cleavage (Miyagishi et al. 2004). In cells, it may even be feasible to convert adenosine (A) to inosine (I) within

dsRNA by RNA editing enzymes (Tonkin and Bass 2003). In case of synthetic siRNA, one could introduce chemical modifications at specific positions within the sense strand of the siRNA molecule to reach the optimal thermodynamic signature. Chemical modification of one of the strands may also be used to either block RISC incorporation of the sense strand or trigger incorporation of the antisense strand. This optimization will also reduce the likelihood of sense-strand-directed off-target effects.

Other aspects may also influence the choice of optimal antivirals, including the effect of RNA secondary structure in the target and the presence of target RNA-associated proteins. When targeting a viral pathogen, it is also particularly important to consider the degree in which the target sequence is conserved among virus isolates. For instance, we selected eight siRNAs against well-conserved and highly accessible domains of the human immunodeficiency virus (HIV)-1 RNA genome, but the majority of these molecules were ineffective, possibly because they did not obey these new rules for optimal siRNA-design (Das et al. 2004).

4
Stable Intracellular Expression of Short Hairpin RNA

The addition of chemically or enzymatically synthesized siRNA to cells is the most convenient way to induce RNAi in the laboratory setting, but stable intracellular expression may be required for several therapeutic applications. The first vectors for the expression of functional siRNA [mostly short hairpin (sh)RNA] were described in 2002 (Brummelkamp et al. 2002; Paddison et al. 2002). Several improvements have been reported, but much optimization is still to be expected. RNA polymerase III (Pol III) promoters have been widely used to express shRNA for silencing a variety of target genes (Brummelkamp et al. 2002), and several modifications of this system were recently published. It was reported that the enhancer from the cytomegalovirus immediate-early promoter (a Pol II unit) can enhance the synthesis of siRNA from a Pol III unit (Xia et al. 2003).

The two different human Pol III promoters that have been widely used naturally encode either a small nuclear (sn)RNA (U6 unit) or part of the RNase P molecule (H1 unit), but more efficient siRNA expression was recently reported for a modified transfer (t)RNA^{met} promoter (MTD unit) (Boden et al. 2003b). Another major improvement is the design of a doxycycline-regulated H1 promoter that allows the inducible knockdown of gene expression by siRNAs (van de Wetering et al. 2003). Similarly, a doxycycline-inducible siRNA expression cassette was inserted in a lentivirus vector (Wiznerowicz and Trono 2003). Alternatively, the ecdysone system has been transplanted onto the U6 promoter for inducible shRNA expression (Gupta et al. 2004). The Cre–loxP recombination system has also been used to switch on shRNA expression, which would

allow regulation in a spatially, temporally or cell- or tissue-specific manner (Kasim et al. 2004). These inducible systems should be particularly useful for the study of proteins that have an impact on cell growth or cell differentiation (e.g. oncogenes and tumour-suppressor genes). The presence of an inverted repeat to encode the hairpin RNA may affect the stability of some vectors, and a convergent transcription unit with two opposing U6 promoters has therefore been designed (Tran et al. 2003). Strategies to express multivalent shRNA constructs have also been described (Anderson et al. 2003), which seems very important to avoid the danger of viral escape (Berkhout 2004). Much remains to be learnt about the shRNA design rules and features that promote efficient shRNA expression modification, intracellular transport, processing, etc. For instance, a detailed mutational analysis elegantly demonstrated that it is possible to introduce multiple G-U base-pairs by mutation of the sense strand (Miyagishi et al. 2004).

5
Inhibition of Viruses by RNAi

Viruses are both inducers and targets of RNA silencing in plants (Vance and Vaucheret 2001). The antiviral capacity of RNA silencing has been used as a tool to generate virus resistance in plants (Lindbo and Dougherty 1992; Smith et al. 2000; Waterhouse et al. 1998). RNAi technology is currently being used to inhibit viral replication in animal cells. Promising results have been obtained with RNAi against several animal viruses both in in vitro and in vivo settings.

The first demonstration of RNAi-mediated inhibition of a human pathogenic virus was reported by Bitko and Barik in 2001 (Bitko and Barik 2001). These authors reported a tenfold inhibition of human respiratory syncytial virus (HRSV) replication in vitro using nanomolar concentrations of synthetic siRNAs that targeted the viral polymerase subunit P and the fusion protein F. Currently, many other studies have described RNAi-mediated inhibition of a large variety of viruses. RNAi-mediated inhibition of HIV-1 has received much attention (see below, Tables 1 and 2). In addition, 17 different RNA viruses, and 10 different DNA viruses have been efficiently targeted by RNAi (Tables 3 and 4). These include important human pathogens such as hepatitis C virus (HCV), dengue (DEN) virus, severe acute respiratory syndrome (SARS) coronavirus, poliovirus, influenza A virus, hepatitis D virus (HDV), human rhinovirus-16 (HRV-16), hepatitis B virus (HBV), herpes simplex virus type-1 (HSV-1), human papillomavirus (HPV), JC virus (JCV), Epstein-Barr virus (EBV), and human cytomegalovirus (HCMV). Other viruses listed in Tables 1 and 4 are: enterovirus 71 (EV71), Semliki Forest virus (SFV), rhesus rotavirus (RRV), flock house virus (FHV), Rous sarcoma virus (RSV), porcine endogenous retrovirus (PERV), foot-and-mouth disease virus (FMDV), murine herpesvirus 68

(MHV68), *Orgyia pseudotsugata* M nucleopolyhedrovirus (OpMNPV), *Autographa californica* nucleopolyhedrovirus (AcNPV), *Microplitis demolitor* bracovirus (MdBV).

Initially, the standard method to induce RNAi towards viruses in mammalian cells was transfection of synthetic siRNAs corresponding to viral sequences shortly before or after a viral challenge. Currently, transient transfection of plasmids that express antiviral shRNAs is also commonly used. Both strategies can result in potent, albeit temporary inhibition of virus replication. In order to obtain long-term virus resistance, researchers have turned to a combined RNAi/gene therapy approach. In this approach, lenti-, retro- or adeno-associated virus (AAV) vectors are used to stably transduce cells with constructs expressing shRNA, resulting in viral resistance. We provide a complete overview of these antiviral studies in Tables 1–4 and discuss the possibility to develop RNAi-based antiviral therapies. The focus will be on HIV-1, and a few RNA and DNA viruses.

5.1 HIV-1 Inhibition by RNAi, Viral Escape and Human Countermeasures

Several studies reported that siRNA can suppress HIV-1 (Capodici et al. 2002; Coburn and Cullen 2002; Hu et al. 2002; Jacque et al. 2002; Lee et al. 2002a; Martinez et al. 2002b; Novina et al. 2002; Park et al. 2002; Qin et al. 2003; Surabhi and Gaynor 2002). There is some evidence suggesting that the genomic RNA present within an infecting virion particle is targeted for destruction, but it appears that new viral transcripts, synthesized from the integrated provirus, are more efficient targets. Most studies used chemically synthesized siRNAs that were transfected into cells either shortly before or after challenge with HIV-1. Despite the transient nature of such a transfection experiment, a single siRNA application is able to achieve relatively long-lasting suppression (Song et al. 2003). Other studies used transient transfection of siRNA-expression vectors. However, the development of efficient vector delivery systems capable of mediating stable siRNA expression in mature T lymphocytes or progenitor stem cells will be a minimal requirement for RNAi to be used as a therapeutic modality against HIV-1. Lentiviral vectors with a Pol III expression cassette are an efficient means to deliver anti-HIV siRNAs into haematopoietic precursor cells. In a recent report, the transduced human cells were allowed to differentiate in vivo in the SCID-hu thymopoiesis mouse model (Banerjea et al. 2003), and the mature T lymphocytes derived from this model resisted HIV-1 infection ex vivo.

Two studies addressed the potency and durability of anti-HIV RNAi approaches. Boden et al. expressed an shRNA against the tat gene in an AAV vector with an H1-promoter (Boden et al. 2003a). Potent inhibition was scored, but an escape virus variant appeared in prolonged cultures. Similar results were described by Das et al. using a lentiviral vector with an H1 unit expressing

Table 1 Inhibition of HIV-1 by RNAi

Target gene	RNAi inducer	Cell type	Fold inhibition of virus replication	Reference
Tat, *Rev*	Intracellular siRNA	293/EcR	10,000	Lee 2002a
LTR, *Vif*, *Nef*	siRNA, shRNA	Magi, PBLs	>20	Jacque 2002
Gag	siRNA	Magi-CCR5, Hela-CD4	>4	Novina 2002
Gag, *Pol*	siRNA	Hos.T4.CXCR4	>10	Hu 2002
Gag, *LTR*	siRNA	U87-$CD4^+$/$CXCR4^+$, $CCR5^+$, 293T	~4	Capodici 2002
Tat+Rev	siRNA	Jurkat, HPBLs	>10	Coburn 2002
Gag, *Env*	500-nt dsRNAs	PBMCs, COS, Hela-$CD4^+$	70	Park 2002
Nef	556-nt dsRNA	MT-4 T, U937	2	Yamamoto 2002
Tat, *Rt*	siRNA	Magi	5–100	Surabhi 2002
Env	siRNA	Cos, Hela-$CD4^+$, PBMCs	~10	Park 2003
Tat	Stable shRNA[a]	macrophages	12	Lee 2003a
Rev	Stable shRNA[a]	$CD34^+$ derived macrophages, T cells in SCID mice	8–16	Banerjea 2003
Tat/*Rev*, *Rev*	Stable shRNA[a]	PBMC	~1,000	Li 2003
Tat	Stable shRNA[b], siRNA and shRNA	293T, H9	33	Boden 2003a
	shRNA, siRNA	293T	14	Boden 2003b
	Stable shRNA[b]	293T, H9	1,200	Boden 2004a
	Intracellular pre-miRNA*	293	25–45	Boden 2004b
Gag	shRNA	293T	275	Pusch 2003
Pol	shRNA	293, Hela	10	Paul 2003
p24	siRNA	MDM	~5	Song 2003
Luc, *GFP*	Stable shRNA[a]	293T, PBMCs	10	Nishitsuji 2004
Nef	Stable shRNA[c]	SupT1	>10	Das 2004
Env, *Tat*/*Rev*, *Rev*, *Nef*, *Pol*	shRNA	293T, CEM	1,000	Scherer 2004
gp41, *Nef*, *Tat*, *Rev*	siRNA	HelaCD4-LTR-β-Gal,HelaCD4, 293T	~200–1,000	Dave 2004

Table 1 (continued)

Target gene	RNAi inducer	Cell type	Fold inhibition of virus replication	Reference
PBS	siRNAs, shRNAs, stable shRNA[b] and HIV vectors[d]	SupT1	~76	Han 2004
Rev	shRNA, stable shRNA[e]	EcR-293, HT1080, 293, CEM	~10	Unwalla 2004
Nef	shRNA	Hela, BHK, Jurkat, MT-4, CRFK	~50	Omoto 2004

All siRNAs were chemically synthesized and transfected into cells unless indicated otherwise. ShRNAs were intracellularly expressed from transfected plasmids under the control of a Pol III promoter (H1 or U6). The fold inhibition of virus production represents the result obtained with the most efficient siRNA or shRNA. [a–e]Stable expression of shRNAs was obtained using: [a] a lentiviral, [b] AAV vector, [c] a retroviral vector, [d] an HIV-vector containing expression cassette for a shRNA against the heterologous PBS sequence, or [e] a lentiviral vector containing a HIV-1 Tat inducible shRNA expression cassette. *Pre-miRNA are shRNAs against HIV made to resemble miRNAs

an siRNA against sequences in the nef gene (Das et al. 2004). The latter study described seven independent HIV-1 escape variants. The combined results convincingly demonstrate that inhibition was potent and sequence-specific, but also that HIV-1 is able to escape from the inhibitory action of a single siRNA. Boden et al. described a single revertant with a point mutation in the target sequence, and Das et al. described a large variety of escape routes (point mutation, double point mutation, partial or complete deletion of the target sequence). A deletion-based resistance mechanism seems impossible in case essential HIV-1 genes or critical sequence motifs are targeted. Thus, one should preferentially target essential sequences that are well conserved among HIV-1 isolates. Interesting targets with relatively little mutational freedom are the multiple overlaps in reading frames within the HIV-1 genome, including a triple overlap (*tag–rev–env*). Ideally, one should target more than one of these essential and well-conserved viral sequences. Such combination siRNA-therapy mimics the successful strategy to combat HIV-1 with multiple antiviral drugs, and should avoid the evolution of escape variants.

We recently discovered an alternative resistance mechanism that is not triggered by mutation of the target sequence. Instead, a mutation in the flanking sequences was selected, which was subsequently shown to induce a conformational change within the target sequence such that it is protected from RISC attack (Westerhout et al. 2005). This finding indicates that it will not be very straightforward to predict viral escape routes. Nevertheless, one could make a first estimation of the chance of viral escape in a therapeutic setting

Table 2 Inhibition of HIV by RNAi-mediated silencing of essential host genes

Target gene	RNAi inducer	Cell type	Fold inhibition of virus replication	Reference
CXCR4/*CCR5*	siRNA	U87-CD4$^+$/CXCR4$^+$, CCR5$^+$	2–3	Martinez 2002b
NF-κB	siRNA	Magi, 293T	5	Surabhi 2002
CD4	siRNA	Magi-CCR5, Hela-CD4	4	Novina 2002
CCR5	siRNA	MDM	∼3	Song 2003
CCR5	shRNA	Magi-CCR5, PBLs	3–7	Qin 2003
CCR5	Stable shRNA[a]	macrophages	∼6	Lee 2003
PARP-1	siRNA	Hela, J111, Magic-5A	∼16	Kameoka 2004
CDK9/CyclinT1	siRNA	Hela,Magi	∼12	Chiu 2004
CXCR4	siRNA	293, HosCD4CXCR4, HosCD4CCR5	2	Zhou 2004
DC-SIGN	Stable shRNA[a]	DCs, Raji B, Hela	6–32	Arrighi 2004
CyPA	snRNA[b] and shRNA	293T, Hela, Jurkat, CEM-SS	∼6	Liu 2004b
SPT5	siRNA	Magi	∼32	Ping 2004

All siRNAs were chemically synthesized and transfected into cells unless indicated otherwise. ShRNAs were intracellularly expressed from transfected plasmids under the control a Pol III promoter (H1 or U6). The fold inhibition of virus production represents the result obtained with the most efficient siRNA or shRNA. [a]Stable expression of shRNAs was obtained using a lentiviral vector. [b]snRNA stands for antisense U7 small nuclear RNAs that disrupt CyPA splicing

with one or multiple siRNAs (Berkhout 2004). If we assume that an essential viral sequence is targeted, deletion is no option. Therefore, one and more likely two nt substitutions are required per 19-nt target sequence to obtain a fair level of resistance (Das et al. 2004). Assuming that 2-point mutations are needed to obtain complete resistance, and further assuming an error rate of the reverse transcriptase polymerase of 2×10^{-5}, the chance of viral escape in a single replication cycle is $19 \times [(2 \times 10^{-5})]^2 = 1.44 \times 10^{-7}$. Studies in the field of drug-resistance indicate that an untreated HIV-infected individual contains an effective viral population size of 10^4–10^5 (Rouzine and Coffin 1999), which means that most 1-nt substitutions will already be present within the viral population. Starting in an untreated patient with a moderate viral load, this means that resistance is likely to occur. Thus, it may indeed be important to consider siRNA combination therapy (SIRCT) (Berkhout 2004). With four effective siRNAs, the chance of viral escape drops to 2.1×10^{-14}. In practice, this

Table 3 Inhibition of RNA viruses by RNAi

Virus	Target gene	RNAi inducer	Cell type	Fold inhibition of virus replication	Reference
HRSV	*P*, *F*	siRNA	A549	~10	Bitko 2001
	P	siRNA	A549, Mice	~5,000	Bitko 2005
	NS1	shRNA	A549, Vero, Mice	~100	Zhang 2005
HPIV	*P*	siRNA	A549, Mice	~100	Bitko 2005
Dengue virus	*Capsid*, *PrM*, *NS5*	~250-nt ssRNA[a]	Mosquitoes, BHK, C6/36	>50	Adelman 2001
	PrM	290-nt dsRNA[b]	C6/36	100	Adelman 2002
	PrM, *E*, *NS1*, *NS5*	77-nt dsRNA	C6/36	10	Caplen 2002
HCV	*NS3*, *NS5B*	siRNA	Huh-7	10	Kapadia 2003
	Capsid, *NS4B*	siRNA	Huh-7.5	~100	Randall 2003
	5′-UTR	siRNA	Huh-7	7	Seo 2003
	5′-UTR, *NS3*, *NS5b*	siRNA, intracell. siRNA	Huh-7	~10	Wilson 2003
	5′-UTR	siRNA, shRNA, intracell. siRNA	Huh-7	~5	Yokota 2003
	5′-UTR, *NS4B*, *NS5A*, *NS5B*	15–40-nt siRNAs, stable shRNA[c]	Huh-7, 9B	20–100	Krönke 2004
	NS5A	siRNA	HepG2, Hep5A	4–5	Sen 2003
	5′-UTR, *core*, *NS3*, *NS5B*	siRNA, shRNA, stable shRNA[d]	Huh-7	~7	Takigawa 2004
Poliovirus	*Capsid*, *3Dpol*	siRNA	Hela S3, mouse fibroblasts	100	Gitlin 2002
EV71	*VP1*, *3D*	shRNA	Hela, Vero	5–10	Lu 2004b
SFV	*Nsp-1,-2,-4*	77-nt dsRNA	C6/36	2	Caplen 2002
RRV	*VP4*	siRNA	MA104	~4	Dector 2002
	NSP4, *VP7*	siRNA	MA104	4–5	Lopez 2005

Table 3 (continued)

Virus	Target gene	RNAi inducer	Cell type	Fold inhibition of virus replication	Reference
FHV	*3′-UTR*	500-nt dsRNA	S2	>100	Li 2002
RSV	*Gag*	siRNA	Chicken embryos, DF-1	5–10	Hu 2002
PERV	*Gag*, *Pol*, *Env*	siRNA, shRNA	293	5–10	Karlas 2004
FMDV	*VP1*	shRNA	BHK-21, Mice	5–10	Chen 2004
	3B, *3D*	siRNA	BHK-21	>10	Kahana 2004
HDV	δ*Ag*	siRNA	Huh-7	5–20	Chang 2003
SARS-CoV	*Pol*	shRNA	Vero	5	Wang 2004b
	Pol	shRNA	Vero-E6, 293, Hela	16	Lu 2004a
	Pol	siRNA	FRhk-4	7–14	He 2003
	Spike	shRNA	293-T, Vero E6	∼10	Zhang 2004b
HCoV-NL63	*Spike*	siRNA	LLC-Mk2	>10	Pyrc in prep
HRV-16	*5′-UTR*, *vp4*, *vp2*, *vp3*, *vp1*, *2A*, *2C*, *3A*, *3C*, *3D*	siRNA	Hela	∼10–20	Phipps 2004
Influenza A virus	*PB1*, *PB2*, *PA*, *NP*, *M*, *NS*	siRNA	MDCK, chicken embryos	200	Ge 2003
	NP, *PA*	siRNA	Mice	56	Tompkins 2004
	NP, *PA*, *PB1*	siRNA, stable shRNA[d]	Mice, Vero	∼10	Ge 2004

All siRNAs were chemically synthesized and transfected into cells unless indicated otherwise. ShRNAs were intracellularly expressed from transfected plasmids under the control a Pol III promoter (H1 or U6). The fold inhibition of virus production represents the result obtained with the most efficient siRNA or shRNA. [a]Intracellular expressed dengue virus ssRNA using Sindbis virus as a vector. [b]290-bp hairpin RNA expressed from a transfected plasmid under the control of hsp 70 promoter. [c,d]Stable expression of shRNAs was obtained using [c] a retroviral vector or [d] a lentiviral vector

Table 4 Inhibition of DNA viruses by RNAi

Virus	Target	RNAi inducer	Cell type	Fold inhibition of virus replication	Reference
HPV-16	*E6*, *E7*	siRNA	CASKi, SiHa	No[a]	Jiang 2002
HBV	*X*, *core*	shRNA	Huh-7, HepG2	20	Shlomai 2003
	Core, *HbsAg*/*Pol*, *X*	shRNA	Huh-7, Mice	>6	McCaffrey 2003
	Core	siRNA	Huh-7, HepG2	~5	Hamasaki 2003
	Core	siRNA	HepAD38, HepAD79	4–50	Ying 2003
	HbsAg	siRNA	HepG2.2.15, Mice	5–100	Giladi 2003
	Core, *S*	siRNA	Mice	3	Klein 2003
	PA, *PreC*, *S*	siRNA	HepG2, 2.2.15	1.7–4.5	Konishii 2004
	hLa[b]	shRNA	HepG2, 2.2.15	19	Ni 2004
	X, *core*, *Pol*, *S*	shRNA	Huh-7	2.5–7	Zhang 2004a
MHV-68	*Rta*, *ORF 45*	siRNA	293T	>43	Jia 2003
Op*M*NPV	Op-*iap3*	511-nt dsRNA	Sf21, Ld652Y	No[a]	Means 2003
AcNPV	*gp64*, *ie1*	619, 451-nt dsRNA	Sf21, *T. mollitor* larvae	>20	Valdes 2003
MdBV	*Glc1.8*, *egf1.0*	289, 359-nt dsRNA	High Five	No[c]	Beck 2003
HSV-1	*gE*	siRNA	HaCaT	~4	Bhuyan 2004
JCV	*VP1*, *Agno*, *T-Ag*	siRNA	SVG-A	10–24	Orba 2004
	Agno, *T-Ag*	siRNA	phFA	~12	Radhakrishnan 2004
EBV	*Zta*	shRNA	NPC-TW01, 293A	11–16	Chang 2004
	LMP-1	shRNA	C666	No[d]	Li 2004a
HCMV	*UL54*	siRNA	U373	~2,000	Wiebusch 2004

All siRNAs were chemically synthesized and transfected into cells unless indicated otherwise. ShRNAs were intracellularly expressed from transfected plasmids under the control a Pol III promoter (H1 or U6). The fold inhibition of virus production represents the result obtained with the most efficient siRNA or shRNA. [a]*E6*, *E7* and Op-*iap3* are nonessential viral genes, but virus production was negatively affected through apoptosis of the host cell. [b]hLa is a host factor that is required for HBV replication. [c]*Glc1.8* and *egf1.0* are nonessential viral genes. [d]*LMP-1* is a non-essential viral gene that plays a role in cell transformation

means that viral escape is impossible as long as viral suppression is complete. Even if several assumptions are wrong, the prospects are favourable that one can achieve effective and long-term viral suppression. An alternative strategy is to target unmutable host-encoded functions that are important for viral replication, but not essential for survival of the host cell (Table 2; Haasnoot et al. 2003).

Effective RNAi-based antiviral therapy is still facing serious technical hurdles, the major one being the delivery of siRNAs into the right cells. Some recent progress has been achieved in this field. Simple conjugates of siRNA and cholesterol, which was chemically linked to the terminal hydrozyl group of the sense RNA strand, were recently reported to trigger tissue delivery (Soutschek et al. 2004). Intravenous injections of the conjugate in mice resulted in uptake into several tissues, including the liver, jejunum, heart, kidneys, lungs and fat tissue. However, many questions remain before this method sees application in humans. For instance, the treatment might require the lifetime use of cholesterol-lowering compounds. Thus, the research on improved delivery systems based on proteins, liposomes or other molecules should continue apace.

Alternatively, RNAi-triggering genes could be transferred into the appropriate target cells. Such a gene therapy protocol seems ideally suited for the treatment of individuals that are chronically infected with HIV-1 and that fail on standard antiretroviral therapy. In chronically infected individuals, HIV-1 infects a significant fraction of the mature T cells each day, leading to cell killing either directly by HIV-1 or indirectly by the HIV-induced immune system. Thus, the preferential survival of even a minority of siRNA-expressing cells will result in their outgrowth over time. One could treat either the mature immune cells from the blood or haematopoietic stem cells from a patient's bone marrow, and put them back into the patient. The latter cells will proliferate into mature T cells and move to the periphery, thus forming a constant supply of cells that resist HIV-1 infection. This means that even a relatively inefficient ex vivo gene therapy protocol could be beneficial. Retroviral and especially lentiviral vectors are frequently used to deliver the siRNA-expression cassette in mammalian cells, although there is concern because the former vector triggered leukaemia in two children in a gene therapy trial (Check 2002). Results are expected soon from the first human trial using lentiviral vectors (Lu et al. 2004d; Lu et al. 2004c).

5.2
In Vivo Evidence for Inhibition of Respiratory Viruses

RNAi-mediated virus inhibition has been studied for a large group of RNA viruses (see Table 3). Recent studies in mice suggest that RNAi holds great promise for the prevention and treatment of infection of the respiratory viruses with an RNA genome such as influenza A virus, human parainfluenza virus

(HPIV) and HRSV (Bitko et al. 2005; Ge et al. 2004; Tompkins et al. 2004; Zhang et al. 2005). Due to limitations of anti-influenza vaccines and drugs, there is a real need for novel strategies to inhibit influenza virus. Worldwide, an estimated half million deaths per year are attributed to influenza virus, and there is the continuous threat of the emergence of a novel pandemic strain. To use siRNA as an in vivo therapeutic, it must be delivered efficiently to the appropriate tissue(s), in this case the lungs. Lungs are perhaps the most readily transfectable organs because they are likely the most vascularized tissue in the body. Furthermore, injected materials will first traverse the capillary beds of the lungs upon intravenous administration. Researchers have used polyethyleneimine (PEI) injected intravenously or intratracheally to deliver siRNAs and a lentiviral DNA vector expressing shRNAs (Ge et al. 2004). PEI is a cationic polymer that has been used to deliver DNA into lung cells. Others have delivered anti-influenza siRNAs intranasally with the cationic transfection reagent Oligofectamine (Tompkins et al. 2004). Reduction of the virus titre in the lungs and lethality was observed when the antivirals were administered either prior or subsequent to virus challenge.

Similarly, replication of HRSV and HPIV in mice could be blocked by intranasal delivery of synthetic siRNAs (Bitko et al. 2005). The authors show that this approach is effective both with and without the use of transfection reagents. Besides synthetic siRNAs, also intranasal administration of plasmids expressing shRNA against HRSV results in a significant decrease of viral titres (Zhang et al. 2005). These findings suggest that low dosages of inhaled or intravenously administered siRNAs/shRNAs might provide an easy and efficient basis for prophylaxis and antiviral therapy against respiratory viruses in human populations.

5.3 Inhibition of HCV by RNAi

HCV is a major cause of chronic hepatitis and hepatocellular carcinoma. Currently, no vaccines are available for HCV, and several groups have used RNAi to target HCV replication. HCV belongs to the family Flaviviridae, and its genome is encoded by a 9.6-kb RNA of positive polarity. Because there is no cell culture system for HCV replication, all studies have used replicon systems in Huh-7 cells as a model for HCV replication. These replicons support HCV RNA transcription and protein synthesis, but do not produce infectious virus. Regions that have been targeted include conserved sequences in the capsid, NS3, NS4A/B, NS5B, and the 5′-UTR (see Table 3). The 5′-UTR of the HCV RNA is a good potential target because it is the most conserved part of the HCV genome that harbours the internal ribosomal entry site (IRES), which is required for translation. Researchers have used siRNAs and shRNAs to block HCV replication (Takigawa et al. 2004). In addition, they looked at virus replication in cells that were transduced with a lentiviral vector to stably express

HCV specific shRNAs. They obtained good inhibition of HCV with shRNAs targeting both *NS3* and *NS5B* with shRNA and in the transduced cells. Poor inhibition was found with shRNA against the 5′-UTR. However, other groups have shown that the 5′-UTR can indeed be a good target (Kronke et al. 2004; Seo et al. 2003; Wilson et al. 2003; Yokota et al. 2003). This again shows that the effectiveness of siRNAs and shRNAs is still difficult to predict.

5.4 Inhibition of Human Coronaviruses by RNAi

Since it became clear that the outbreak of SARS beginning 2003 is caused by the virus currently known as SARS-coronavirus (SARS-CoV), researchers have tried to find cures for this new virus. It was shown that both shRNAs and siRNAs could efficiently block SARS-CoV replication in tissue culture systems (He et al. 2003; Lu et al. 2004a; Wang et al. 2004b; Zhang et al. 2004b). In these studies, the main target was the polymerase gene, whereas one paper describes inhibition of SARS-CoV by targeting the spike protein, which is essential for particle formation and entry (Zhang et al. 2004b). Additionally, replication of the newly discovered human coronavirus NL63, HCoV-NL63 (Van der Hoek et al. 2004), could also be inhibited by siRNAs that target the spike gene (Pyrc et al. in preparation). Because SARS is a disease of the upper airways and lungs, it could be relatively easy to administer therapeutic siRNAs. For influenza virus, HRSV and HPIV, it has been shown that virus replication in the lungs of mice can be inhibited by intravenous or intranasal administration of siRNAs/shRNAs (Bitko et al. 2005; Ge et al. 2004; Tompkins et al. 2004; Zhang et al. 2005). Following a similar route, siRNAs against SARS-CoV might be effective as a new antiviral therapeutic.

5.5 Inhibition of HBV by RNAi

In contrast to RNA viruses, RNAi against DNA viruses targets only the viral mRNA transcripts, but not the viral genome. This suggest that RNAi against DNA viruses might be less effective than RNAi against RNA viruses. However, the published data on RNAi mediated inhibition of DNA viruses indicate that this is not the case. HBV is a member of the Hepadnaviridae and its genome is a 3.2-kb double-stranded circular DNA. HBV infection can cause liver cirrhosis, which may ultimately lead to hepatocellular carcinoma. Although vaccines have been developed that can prevent infection, HBV remains a serious health problem in many countries. A number of studies show that RNAi induced by synthetic siRNAs can block HBV both in cell culture and in mouse model systems (Hamasaki et al. 2003; Klein et al. 2003; Konishi et al. 2003; Ying et al. 2003). For instance, Giladi et al. showed a 5–12-fold inhibition of HBV replication in mice that have been treated with synthetic siRNAs

targeting the small HBV surface antigen, HbsAg (Giladi et al. 2003). Earlier, a 6-fold inhibition of HBV in the liver of mice was obtained by intravenous injection of plasmids expressing shRNAs against the core, HbsAg/Pol and X gene (McCaffrey et al. 2003). Possibly, a similar approach could be used in infected patients to lower virus titres. Besides targeting the viral RNA, it is also possible to inhibit HBV replication by targeting the mRNAs of cellular factors that are required for virus replication. Ni et al. have shown that targeting the hLa protein results in a decrease in virus replication (Ni et al. 2004). Additionally, it has been shown that RNAi can be used to prevent HCV- or HBV-induced disease of the liver by silencing the expression of the cellular Fas gene. During HCV or HBV infection Fas-mediated apoptosis of hepatocytes is triggered as a self-destructive inflammatory response of the liver. Silencing Fas expression with synthetic siRNAs blocks this reponse (Li et al. 2004a).

5.6 Inhibition of DNA Viruses by RNAi

HSV-1 is a large DNA virus that infects epithelial and neuronal cells. Bhuyan and co-workers used siRNAs against glycoprotein E (gE) to inhibit HSV-1 replication. The gE is important for cell-to-cell spread and evasion from complement and antibody responses, and silencing the expression of gE resulted in a fourfold inhibition of HSV-1 replication (Bhuyan et al. 2004). An 11- to 16-fold inhibition of the gamma herpesvirus EBV was obtained with shRNA against the essential viral gene Zta (Chang et al. 2004). Zta is involved in the reactivation of EBV and important for expression of lytic genes and viral DNA replication. In addition to targeting EBV replication, one report describes inhibition of an EBV oncogene to inhibit the pathogenic effects of EBV infection (Li et al. 2004a). EBV is associated with the development of highly metastatic nasopharyngeal carcinoma (NPC). Important in the development of NPC is the viral latent membrane protein-1 (LMP-1), which is involved in cell transformation and tumour metastasis. Suppression of LMP-1 expression by RNAi resulted in altered cell motility, surface adhesion and transmembrane invasion ability, suggesting that RNAi can be used to inhibit the metastatic potential of the EBV-positive carcinoma cells.

Two studies have used RNAi to inhibit the small DNA virus human polyomavirus JCV (Orba et al. 2004; Radhakrishnan et al. 2004). JCV can cause progressive multifocal leukoencephalopathy (PML) in patients with impaired immune systems. As such, PML has become a major neurologic problem among patients with AIDS. In both studies, synthetic siRNAs were used to target the VP1, Agno and T-Ag genes, resulting in 10- to 24-fold inhibition of virus replication in vitro.

6
Off-Target Effects

An important question mark concerning RNAi-therapy is its specificity. The introduced siRNA may negatively affect the production or activity of endogenous RNAi pathways that are involved in the regulation of cellular gene expression. This is particularly important because there is growing evidence for the biological significance of RNAi in development (Bernstein et al. 2003; Wienholds et al. 2003). It also needs to be proved that RNAi will not cross-silence cellular mRNAs with a sequence motif that resembles the actual viral target. Genome-wide expression profiling yielded promising results (Chi et al. 2003; Li et al. 2004b; Semizarov et al. 2003), but other studies reported that siRNA-treatment can cause changes in the expression of dozens of cellular genes that are not directly targeted (Jackson et al. 2003; Persengiev et al. 2004; Scacheri et al. 2004). However, microarray expression profiling may provide a very sensitive readout compared to a typical functional screen. With shRNA molecules it seems critical to use hairpins with a stem of 19 base-pairs or less in order to avoid induction of the interferon response and cytotoxic effects (Fish and Kruithof 2004).

siRNAs with imperfect complementarity can silence genes by repressing mRNA translation while not affecting the levels of mRNA, and up to three or four mismatches are tolerated in this system (Saxena et al. 2003). This finding, combined with the observation that the G-U wobble is recognized as a regular base-pair, emphasizes the possibility of off-target silencing by siRNAs. Most of these studies were performed with synthetic siRNA, of which the concentration added to cells may also be a critical factor (Persengiev et al. 2004). Intracellularly expressed shRNA may affect cellular genes by other means (Bridge et al. 2003). It is therefore obvious that the use of RNAi-based therapeutic agents requires a further demonstration of the absence of adverse effects on cell physiology.

Another issue is whether siRNA will induce the interferon system, which causes cells to shut themselves down in response to invading RNA viruses. In contrast to plants and *C. elegans*, transfection of dsRNA longer than 30 base-pairs into mammalian cells induces this interferon pathway via dsRNA-dependent protein kinase (PKR). The induced antiviral response involves non-specific degradation of RNA and generalized inhibition of translation. siRNA was supposed not to be detected by the radar of the interferon system, but this may not be completely true. Two recent papers examined the effect of siRNAs on the interferon system (Bridge et al. 2003; Sledz et al. 2003). Both studies reported nonspecific changes in expression of interferon-stimulated genes (Moss and Taylor 2003). One study reported activation of the PKR by a synthetic 21-bp siRNA (Sledz et al. 2003), but this finding is not consistent with previous results (Manche et al. 1992). A detailed characterization of the minimal RNA motif for PKR activation revealed the following characteristics:

a hairpin with a (possibly imperfect) 16-bp stem flanked by 10- to 15-nt single-stranded tail (Zheng and Bevilacqua 2000). These results indicate that siRNA is not a PKR-activator, but does not rule out a role for PKR in RNAi biology.

It was reported that certain genes of the interferon system are activated upon siRNA-introduction into cells, thus providing another serious warning that off-target effects may be more common than initially anticipated (Sledz et al. 2003). Furthermore, dsRNA can activate several protein kinases such as p38, c-Jun N-terminal kinase (JNK)2 and I kappa B kinase (IKK) in addition to PKR. Induction of these signalling pathways can alter gene expression by regulating the activity of transcription factors such as nuclear factor (NF)-κB, interferon regulatory factor (IRF)-3, and ATF-1 (Williams 1999). Therefore, caution must be exerted in the interpretation of data from experiments using RNAi technology for suppression of specific genes. It will be important to first add to our basic understanding of how siRNA exactly works before rushing into clinical trials. Among other things, these experiments need to be repeated in animals, and that is exactly what was done very recently (Heidel et al. 2004). Regardless of the injection method into mice, siRNA failed to trigger a strong type I interferon response, unlike the long double-stranded control RNA poly(I:C). The siRNAs were shown to silence genes in a sequence-specific manner, demonstrating that they reached the intracellular target. The absence of both an interferon and inflammatory response in vivo is good news for using siRNAs therapeutically.

7
Viruses Utilize RNAi in Their Replication Strategy

Alternatively, viruses could exploit RNA silencing to control the expression of genes of viral or host origin. The first example was recently provided for EBV, a large DNA virus of the herpes family that preferentially infects human B cells (Pfeffer et al. 2004). When the small RNAs from a latently EBV-infected Burkitt's lymphoma cell line were cloned, 4% of them originated from two regions of the EBV genome. A computational method was used to identify potential targets of these EBV-encoded miRNAs. Among the predicted targets were regulators of cell proliferation, apoptosis, transcriptional regulators and components of signal transduction pathways. Although these targets should be verified experimentally, it is striking that several of these genes have more than one binding site for a particular EBV-miRNA. Degradation of a cellular DNA polymerase was demonstrated experimentally (Pfeffer et al. 2004). Furthermore, the expression of the EBV-miRNAs was shown to differ in the lytic versus latent stage, suggesting tight regulation during viral infection. The viral miRNAs could be involved in tumour formation and may explain how EBV hides so well. Other members of the herpesvirus family and other viruses with

a large DNA genome could encode miRNAs in order to exploit RNA silencing for the regulation of host and viral gene expression.

Both a theoretical and an experimental study recently identified several potential miRNAs encoded by the HIV-1 RNA genome (Bennasser et al. 2004; Omoto et al. 2004). In addition, Bennasser et al. (2004) identified several corresponding cellular RNAs that could potentially be targeted by these viral miRNAs.

7.1 Viroids Also Utilize the RNAi Mechanism

Viroids are single RNA molecules that have no protective protein coat and that do not encode protein. Viroids can cause severe disease in plants, and several hypotheses have been proposed to explain disease induction in the absence of any viral protein. Viroids could interrupt the function of an unknown host cell factor or use small regulatory RNAs to influence host gene expression. Now it appears that viroid pathogenicity may involve the RNA silencing pathway. Wang et al. reported that engineered tomato plants, which express virion-derived non-infectious hairpin RNA, had symptoms mimicking those of viroid infection (Wang et al. 2004a). Much remains to be learned, for instance how nuclear-replicating viroids can exploit the RNAi machinery that is located in the cytoplasm, and which cellular mRNA is targeted. Nevertheless, the EBV and viroid examples underscore the putative pathogenic function of virus-derived small RNA molecules.

8 RNAi Versus the Antiviral Interferon System

Assuming that RNAi acts as an antiviral mechanism in humans, one would predict that human viruses have developed countermeasures, although it has been questioned whether RNAi plays a major role in the antiviral defence in vertebrates (Saksela 2003). However, unlike plants and invertebrates, vertebrates also have the interferon system that responds to dsRNA by inducing the synthesis of a large group of proteins that have a general inhibitory effect on virus multiplication. The best-characterized interferon-induced genes encode PKR kinase and the 2′-5′oligo A synthetase enzymes, both of which are activated in response to dsRNA (Goodbourn et al. 2000). Activated PKR causes an inhibition of protein synthesis by phosphorylation of eIF2, and 2′-5′oligoA synthetase induces general RNA degradation via activation of RNase L, leading to ultimate cell death via apoptosis. Thus, mammals with their adaptive immune system have already defence mechanisms that respond to dsRNA. However, the discovery that RNAi is triggered by siRNAs (Caplen et al. 2001; Elbashir et al. 2001), which are too short to efficiently activate the interferon response pathway (Moss and Taylor 2003), suggested that RNAi may also play a role

in the cellular defence against infection by human viruses. Thus, human cells may have two alternative pathways to combat dsRNA; long dsRNA activates the interferon response pathway, whereas short dsRNA (< 30 bp) activates RNAi.

9
Adenovirus VA RNAs as Suppressor of the RNAi and Interferon Systems

It is well established that most mammalian viruses have evolved defence strategies to suppress the negative effects that the interferon response pathway has on virus multiplication. This is of vital importance for the capacity of a virus to multiply successfully. Numerous viruses encode proteins or decoy RNAs that inhibit the activity of PKR by a surprisingly large range of different strategies (Gale and Katze 1998). For example, human adenovirus type 5 (ad5) encodes two approximately 160-nt non-translated RNA polymerase III transcripts; the highly structured VA RNAI and VA RNAII (Mathews 1995). VA RNAI has been shown to stimulate protein synthesis in infected cells and in transient transfection assays by blocking activation of the interferon-induced antiviral defence system (Kitajewski et al. 1986; Svensson and Akusjarvi 1984). VA RNAI binds to PKR and acts as a competitive inhibitor (Gale and Katze 1998), thus preventing viral dsRNA that is produced by symmetrical transcription of the viral DNA from activating PKR.

We recently demonstrated that human adenovirus also inhibits the RNAi machinery at late times of infection (Andersson et al. 2005). The suppression of RNAi results from a virus-induced block of the two key enzymatic activities in RNAi, Dicer and RISC. We further showed that VA RNAI and VA RNAII have the capacity to suppress RNAi in transient transfection experiments. Mechanistically, the VA RNAs appear to block RNAi by acting as competitive substrates that squelch Dicer. Since VA RNAs are expressed at copious amounts at late times of infection [up to 10^8 copies/cell, (Soderlund et al. 1976)], one would expect that they are produced in great excess over any aberrantly formed dsRNA. Therefore, a simple competitive inhibition for binding to Dicer would be sufficient to explain the inhibitory effect of the VA RNAs on RNAi. VA RNAs might function as suppressors of RNAi because they form highly structured motifs with imperfect stems that resemble precursors to miRNA and therefore might sequester Dicer by acting as competing substrates, or pseudo-substrates. The finding that VA RNAI and VA RNAII are indeed processed by Dicer into siRNA both in vitro and during a lytic infection supports this model and shows that the VA RNAs can interact with Dicer. Unlike Dicer inhibition, the VA RNAs are not required for the observed inhibition of RISC during infection. Most likely the inhibition of RISC requires another, yet-to-be-identified, viral factor. Recently, Lu et al. (2004) published similar results for adenovirus VA RNAI (Lu and Cullen 2004). Collectively, these results suggest that the adenovirus VA RNAs antagonize the cellular defence pathways directed against both long

(interferon-induced) and short (RNAi-induced) dsRNA by inactivating two key enzymes, PKR and Dicer.

These findings have an impact on strategies that use viral vectors for RNA silencing purposes. The possible existence of viral suppressors of RNAi should have consequences on how adenoviral vectors, and potentially other viral vectors, are designed to create optimal vectors for siRNA delivery to target cells. For instance, the VA RNAs are expressed not only from replicating adenoviral vectors, but also from non-replicating adenoviral vectors. Thus, one would expect that VA RNA might negatively affect the efficiency of adenovirus-delivered shRNA. Further, it is possible that an adenoviral vector may alter cellular gene expression as a result of competition between VA RNA and cellular miRNAs, as has been seen in virus-infected plants (Kasschau et al. 2003).

10 The Future of RNAi Therapeutics

One of the most important consequences of the RNA silencing revolution is the ability to use the RNAi pathways to determine gene function and to apply RNA silencing as a tool in agriculture and medicine, e.g. to protect against viral infections. Indeed, RNAi therapy is expected to make its way towards clinical trials in the near future. The interplay between RNAi and viruses is very complex as viruses can be inducers, suppressors and actual targets of the RNAi silencing mechanism. There is an interesting and strong argument that RNA was the primordial biopolymer of life because of its multi-functionality (both enzyme and replicon). A relatively small cadre of scientists has always been dedicated to studying RNA, but RNAi has brought about a real RNA revolution that seems to have converted all scientists.

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