

## Minireview

## The therapeutic potential of RNA interference

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**Abstract** In recent years, we have witnessed the discovery of a new mechanism of gene regulation called RNA interference (RNAi), which has revitalized interest in the development of nucleic acid-based technologies for therapeutic gene suppression. This review focuses on the potential therapeutic use of RNAi, discussing the theoretical advantages of RNAi-based therapeutics over previous technologies as well as the challenges involved in developing RNAi for clinical use. Also reviewed, are the *in vivo* proof-of principle experiments that provide the preclinical justification for the continued development of RNAi-based therapeutics. © 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

**Keywords:** Gene suppression; Drug development; Delivery; Safety; RNA interference

## 1. Introduction

The pharmaceutical industry has an ongoing interest in developing technologies for therapeutic gene suppression. Previous efforts focused on sequence-specific RNA knockdown technologies such as antisense oligonucleotides and ribozymes. While some success has been achieved with these drug platforms [1], technical issues regarding delivery, stability, off-target effects, and effective target sequence selection have slowed the development of efficacious clinical drugs. In recent years, however, we have witnessed the discovery of a new mechanism of gene regulation called RNA interference (RNAi), which has revitalized interest in the clinical development of nucleic acid-based gene inhibition approaches. This review focuses on the potential therapeutic use of RNAi. After a brief introduction to RNAi, we discuss the theoretical advantages of RNAi-based therapeutics over previous technologies and then temper that optimism with a discussion of the challenges involved in developing RNAi for clinical use. Finally, we review proof-of principle experiments that have demonstrated the clinical potential of RNAi, highlighting some of the *in vivo* successes that provide the preclinical justification for the continued development of RNAi-based therapeutics.

RNAi is a naturally occurring biological process that is highly conserved among multicellular organisms diverse as plants, worms, yeast, and humans. It refers to a two-part intracellular pathway in which precursor double-stranded RNA molecules present in the cell are first processed by the dicer endonuclease into short 21–23 nucleotide fragments containing 2 nucleotide

single-stranded 3' overhangs on each strand. These effector RNAs, called short interfering RNAs (siRNAs), then become incorporated into an RNA-inducing silencing protein complex (RISC) in which one strand of the unwound siRNA acts as a guide sequence to target the cleavage of homologous RNAs [2–4]. In plants, RNAi plays a role in cellular defense, protecting the cell from inappropriate expression of repetitive sequences, transposable elements, and virus infections (reviewed in [5]). In addition to evidence that similar defense functions may still be active in mammalian cells [6,7], an increasing number of short RNA molecules are being found encoded in the mammalian genome. These endogenous RNAs, or microRNAs (miRNAs), are also processed by dicer into siRNA effectors, and are proving to regulate the expression of genes involved in a variety of cellular processes such as proliferation, apoptosis, and differentiation (reviewed in [8–10]). Importantly, when siRNA molecules are chemically synthesized and exogenously introduced into mammalian cells, they become incorporated into the cellular RISC complex and mediate the degradation of RNAs to which they are homologous [2–4].

The ability to tap into this native pathway has been recognized as one of the most exciting biotechnology advances in the last decade. Indeed, RNAi has revolutionized biology research, including drug target discovery, by allowing for rapid identification and validation of gene function. While this alone justifies RNAi being named the 'Breakthrough of the Year' in 2002 by *Science* magazine, many are hopeful that the greatest contribution of RNAi is yet to come, that it will serve as a means of sequence-specific therapeutics against a wide range of diseases. As such, several companies are focusing on the development of RNAi-based therapeutics (Table 1) [11].

## 2. Advantages of RNAi therapeutics

### 2.1. Specificity

One of the potential advantages of sequence-based gene suppression technologies is the ability to design precisely targeted therapeutics for almost any gene, regardless of the function of the gene product, whether that function is clearly defined, and in the absence of any protein structure information. Particularly in the fields of oncology and genetic neurological disorders where disease is often caused by a dominant mutation in a single allele, this would offer the opportunity to selectively inhibit expression of only the defective gene. However, being able to identify an effective target sequence in which a single polymorphism can be distinguished is not trivial. Impressively, although our knowledge of optimal siRNA target selection is still limited, RNAi activity is specific enough that allele-specific

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Table 1  
Companies developing RNAi therapeutics

Company name	Primary areas of interest
Acuity Pharmaceuticals	AMD; diabetic retinopathy
AGY Therapeutics	RNAi in neurons and glial cells
Alnylam Pharmaceuticals Inc.	AMD; Parkinson's disease, respiratory syncytial virus; cystic fibrosis; influenza; spinal cord injury
Atugen AG	Metabolic disease; cancer ocular disease; skin disease
Benitec Australia Limited	Hepatitis C virus; HIV/AIDS; cancer; diabetes/obesity
Calando Pharmaceuticals	Nanoparticle technology
Cytrx Corporation	Diabetes/obesity; ALS; cytomegalovirus retinitis
Devgen	Diabetes/obesity; arrhythmia
Genesis R&D	Allergy
Genta Incorporated	Cancer
International Therapeutics	HIV; Hepatitis B virus
Intradigm Corporation	Cancer; SARS; arthritis
Nucleonics, Inc.	Hepatitis B Virus; Hepatitis C virus
Sirna Therapeutics, Inc.	AMD; Hepatitis C virus; asthma; diabetes; cancer Huntington's disease; hearing loss

AMD, age-related macular degeneration; SARS, severe acute respiratory syndrome coronavirus; ALS, amyotrophic lateral sclerosis.

silencing has already been demonstrated for several prominent cancer and neurological targets [12–17].

## 2.2. Potency

While it can be difficult to directly compare the efficiency of gene suppression technologies due to differences in the rules for optimal design and target sequence selection, several studies have supported the conclusion that RNAi-mediated inhibition is more potent than that achieved with antisense oligonucleotides even in cases where site selection was optimized for antisense effectiveness (reviewed in [18]). This is also empirically evident by the remarkable speed at which RNAi techniques have been implemented in scientific research, and the number of successful RNAi-based experiments already published. It is speculated that this advantage is derived from the fact that RNAi is an innate biological response, and hence represents a more natural strategy for manipulating gene expression. In any event, it means that physiologically beneficial silencing can be achieved with lower concentrations of effector molecules [19–23] (reviewed in [18,24]).

## 2.3. Versatility

Perhaps due to its superior potency, it is also relatively easy to identify effective RNAi target sites. Although the rules for optimal RNAi effectiveness are still being determined, when basic parameters regarding CG content and the composition of the 3' overhangs are met, then a high percentage of potential targets screened usually prove to be functional [21,23,25–28]. On the other hand, it is significantly more difficult to identify efficient antisense oligonucleotide target sequences [21,23,29] (Uprichard, unpublished data) and ribozyme target selection is limited by the availability of particular sequence motifs required for cleavage [30]. The versatility and ease with which RNAi-mediated inhibition can be induced means that multiple sequences within an individual gene, or a group of genes, can be targeted simultaneously, more readily providing the benefit of combination therapy.

## 3. Challenges of RNAi therapeutics

### 3.1. Delivery

The problem of delivery is not unique to RNAi therapeutics, but it is by far the major obstacle to the clinical use of RNAi-based drugs.

**3.1.1. RNAi effectors.** RNAi effectors can be delivered to cells using two different approaches. In one case, siRNAs are synthesized in the laboratory to be delivered as a “drug”. The second option is a gene therapy approach in which DNA encoding short hairpin RNA (shRNA) expression cassettes are delivered to cells to allow for intracellular expression of shRNAs, which are then processed into active siRNAs by the host cell [31,32]. The potential advantages of the latter DNA approach include the inherently higher stability of transiently delivered plasmid DNAs and the fact that this strategy consists of an intracellular amplification step in which large amounts of shRNAs can be synthesized from each individual template. Additionally, it is also possible to deliver DNA expression vectors stably, either by integration into the genome or in self-replicating episomal form, which theoretically could allow for constitutive expression of the shRNA cassette.

**3.1.2. Local delivery.** Based on the precedent of successful local administration of antisense drugs to the eye [33], initial clinical trials for RNAi-based treatment of age-related macular degeneration are all using local injection of siRNAs directly into the eye [34]. Other promising local routes are intranasal administration for pulmonary delivery [35–37] and direct delivery into the central nervous system [38–40]. Nonetheless, for RNAi therapeutics to overcome the impediments of the gene silencing technologies that came before it, effective systemic delivery and subsequent targeting to the correct cells must be achieved.

**3.1.3. Systemic delivery.** Optimizing systemic delivery requires stabilization of the siRNA, targeting of the effector to the correct tissue, and facilitation of cellular uptake. Thus far, approaches to improve stability and cellular uptake of siRNA drugs include many of the same strategies employed for antisense oligonucleotides such as direct chemical alteration of the nucleic acid [41,42] and various methods of packaging the effector into protective particles [41,43–47]. To target the effector to particular cell types, different ligands [48–50] and antibodies [51] are being incorporated into/conjugated to the RNAi effector, but continued work is needed to achieve the efficiency required.

Notably, the use of viral vectors may be useful for systemic delivery of RNAi effectors. Unfortunately, although viral vectors can provide the excellent tissue-specific tropism and transduction efficiency needed for clinical delivery, each type of viral vector brings with it a unique set of risks and safety concerns (reviewed in [52]). Despite setbacks encountered in past

gene therapy clinical trials, however, the need for and benefits of RNAi therapeutics are compelling and in some cases may outweigh the risks. Hence, both lentivirus and adeno-associated virus (AAV) vectors are being considered for clinical delivery of shRNAs [53,54].

### 3.2. Safety

The aim of any therapeutic is to maximize the ratio of desired effects to undesired effects. In some cases, such as chemotherapy, interferon treatment, and highly active antiretroviral treatment, the ratio is not ideal and a significant degree of toxicity is associated with treatment. While RNAi has the capacity to provide better gene targeting specificity, the exposure of cells to any exogenous molecule (siRNA or delivery agent) has the potential to perturb normal cellular functions and needs to be carefully controlled.

**3.2.1. Off-target effects.** While specificity is one of the greatest advantages offered by RNAi, off-target effects are still a potential problem, particularly because the parameters that determine the minimum level of homology required for siRNAs to mediate inhibition of a gene are not yet known. Depending on the sequence of an siRNA, as few as 11 base pairs of homology with a single-stranded RNA has been found to result in inhibition [55]. Importantly, the wealth of searchable genetic information now available provides a valuable tool that can be used to try to avoid inadvertent off-target homology, but rigorous empirical screening of all potential siRNA effectors will continue to be necessary to limit off-target effects.

**3.2.2. Non-specific effects.** The issue of non-specific toxicity is twofold for RNAi because both the delivery vehicle and the siRNA effector itself may elicit unexpected cellular responses. Firstly, it has been found that some cationic liposomes used to deliver siRNAs might induce or potentiate interferon responses [56,57]. Likewise, any viral vector that might be used to transport shRNA expression cassettes into cells could stimulate an undesired vector-specific immune response. Secondly, the nature of siRNA effectors themselves may trigger the induction of the double-stranded RNA cellular defense mechanism. Although interferon induction might be beneficial for therapy in some instances, uncontrolled induction of this innate defense mechanism can be cytotoxic, and is therefore a concern. Initial gene expression profiling by different laboratories provided conflicting results regarding whether or not interferon is induced in response to siRNAs [55,58–61]. Recent studies, however, have started to systematically analyze these differences revealing that while certain siRNAs do induce an interferon response, others do not (reviewed in [62]). For example, Hornung et al. [63] have identified a 9 base pair “danger motif,” which in the context of an siRNA effector induces interferon signaling. Additionally, the structure of introduced siRNA molecules can dictate whether or not interferons are induced, as Kim et al. [64] have reported that the presence of a 5' phosphate can trigger interferon induction. Consequently, careful design and screening of each candidate siRNA and delivery vehicle will be necessary to try to identify and minimize all potential adverse effects.

**3.2.3. Saturation of RNAi machinery.** While the ease and efficiency of siRNA inhibition may be derived from the fact that these exogenously added molecules are usurping a natural cellular pathway, a growing safety concern is that this could interfere with the regulatory functions of endogenous miRNAs,

which are known to share some of the same processing machinery [65]. Evidence suggests that when excess siRNAs are delivered to mammalian cells, the intracellular RNAi processing machinery can become saturated. This conclusion is drawn from studies that demonstrate competition between co-delivered siRNAs targeting two different genes [36] and inhibition of siRNA function by a co-delivered scrambled siRNA [66,67]. While we are just beginning to appreciate the diverse roles miRNAs play in mammalian cells, accumulating data indicates that they are involved in many critical aspects of gene regulation (reviewed in [8–10]). Furthermore, alterations in the expression of miRNAs or their processing machinery have recently been implicated in several neurological diseases [68–70] and cancers [70–77]. In some instances over expression of miRNAs has been found to be oncogenic [73,76,77], but in other cases reduced miRNA expression is observed in cancer cells [71,74,75]. It will therefore be important to assess the long-term consequences of RNAi therapy, in order to determine if miRNA functions are negatively affected. To overcome this problem, it might be necessary to analyze different doses of siRNAs to determine the maximum capacity of the intracellular siRNA processing machinery in different cell types.

### 3.3. Efficacy

A wide variety of issues will ultimately determine the efficacy of particular RNAi-based therapeutics.

**3.3.1. Resistance.** Ironically, the exceptional sequence-specificity of RNAi is also viewed as one of its potential weaknesses. This problem is often cited when discussing the use of RNAi as an antiviral agent as it has been demonstrated that both RNA and DNA viruses can rapidly generate RNAi escape mutations [78–82]. This problem will probably necessitate the use of RNAi in combination therapy approaches, including multiple RNAi target sequences and/or other synergistic antivirals, such as small molecule inhibitors. Another possible issue regarding viral resistance is the discovery that analogous to plant viruses (reviewed in [5]), some mammalian viruses, such as Nodamura virus and human immunodeficiency virus (HIV), encode genes that may interfere with RNAi silencing [6,7]. Notably however, the effectiveness of these virally encoded genes in preventing therapeutic RNAi inhibition remains to be determined. Resistance of particular RNAs to RNAi-mediated degradation has also been observed in cases where accessibility of the target sequence was restricted. For cellular mRNAs, the structure of the target RNA has been found to affect silencing efficiency [83,84]. For viral RNAs, resistance can also be related to intracellular location and/or nucleocapsid association of genomic RNA molecules [85–87].

Resistance may also be a fundamental problem for the treatment of some cancers. As altered expression of miRNAs and their processing enzyme, dicer, have recently been found to be associated with different types of cancer [70–77], it is possible that the processing machinery required for siRNA activity is not functionally normal in some tumor cells. Hence, depending on the nature of the oncogenic defect, it is possible that therapeutically delivered siRNAs may not be as effective in these cells. On a positive note, preliminary studies have demonstrated the effectiveness of siRNAs in multiple cancer models. Yet, the role miRNAs play in oncogenesis and how this might impact siRNA processing is an area that needs to be further investigated.

**3.3.2. Incomplete inhibition.** Although RNAi is more potent than other gene silencing methods [19–23] (reviewed in [18,24]), when designing RNAi-based therapeutics it is necessary to consider the quantitative limitations inherent in this technology. Specifically, RNAi knocks-down gene expression, but generally does not eliminate it. For some conditions, partial down regulation of a pathogenic gene appears to be sufficient to produce clinically relevant improvement, however this is not “cure”. In the case of genetic diseases, a “cure” is not necessarily expected, but for other conditions such as cancer or infectious disease, the ideal goal of therapy would be to rid the patient of the disease. This limitation has been illustrated in several xenograph mouse tumor models in which siRNA silencing of oncogenes *in vivo* has been shown to slow the rate of tumor progression and prolong survival, but the tumors were not cleared [88,89]. Hence, in many cases, RNAi treatment strategies may only be effective if designed in the context of a synergistic combinatorial therapy approach.

**3.3.3. Duration.** Also related to efficacy is the duration of the therapeutic effect. When delivered as a drug, siRNAs and shRNA-expressing DNA templates function transiently. When transfected into cells in culture, the inhibitory effect usually peaks at day 2 post-transfection and fades thereafter. Alterations in siRNA stability (see below) may prolong this effect, but in the absence of any endogenous RNAi mechanism for amplification or spread in mammalian cells, the activity of these delivered nucleic acids is expected to remain transient in nature. If the delivered siRNAs are part of an acute curative treatment, then transient activity might be sufficient, but in cases where RNAi suppression is part of a long-term treatment protocol it will require frequent dosing probably at significant cost. Notably, the desire to develop

RNAi-based therapeutics for chronic conditions, such as genetic defects, is one of the main motivations for developing gene therapy delivery techniques, which presumably would allow for constitutive intracellular expression of the therapeutic shRNA.

**3.3.4. Stability.** Although some data indicate that siRNAs are more stable in serum and mammalian cells than antisense oligonucleotides and ribozymes [19], multiple approaches are being investigated to alter the pharmacokinetic properties of siRNAs to increase their half-life *in vivo*. Not only are protective vehicles, such as liposomes, being designed to escort and target intact siRNA effectors for cellular uptake, but chemical modification of the siRNAs is also being investigated as a means of directly stabilizing the molecules. Importantly, several groups have shown that inhibition of gene expression by siRNAs is compatible with a broad spectrum of chemical modifications [26,42,90–93]. Most recently, Morrissey et al. demonstrated that a combination of direct modifications improved stability, and thus *in vivo* efficacy of intravenously administered siRNAs [40]. However, as determined by the authors, less than 1% of the injected siRNA reached their target organ indicating that modifications to improve targeting and/or cellular uptake will also be necessary.

Importantly, despite these challenges, a wide variety of disease-relevant genes have been specifically inhibited with siRNAs and shRNAs *in vitro* and in small animal models (Tables 2–4). Additionally, planned and ongoing clinical trials will soon provide valuable information regarding efficacy, dosing, delivery, and safety, which should be useful in the continued advancement of RNAi-based drugs and in the analysis of issues such as compliance which factor into the success of any therapeutic regimen.

Table 2  
Inhibition of viral gene expression and replication by RNAi

Virus	In vitro	In vivo			Reference	
	Reference	Model system	Delivery	Reference		
			Vehicle	Route	Time of treatment	
West Nile Virus	[95]	Mouse infection	None	hd	Prophylactic (at 4 h)	[96]
Hepatitis C Virus	[97]	Reporter transcript	None	hd	Co-transfection	[98]
Coxsackievirus B3	–	Mouse infection	None	hd	Therapeutic (at 6 h)	[99]
RSV <sup>a</sup>	[87]	Mouse infection	None/TransIT	i.n.	Prophylactic (at 4 h)	[36]
					Therapeutic (at 1–3 days)	
Parainfluenza	–	Mouse infection	None/TransIT	i.n.	Prophylactic (at 4 h)	[36]
Influenza	[95,100]	Mouse infection	PEI-complex lentivirus	i.v./i.n.	Prophylactic (at 12 h)	[101]
					Therapeutic (at 5 h)	
		Mouse infection	Oligofectamine	hd/i.n.	Prophylactic (at 24 h)	[102]
Hepatitis B virus	[103–105]	Hydrodynamic	None	hd	Co-transfection	[106]
		Hydrodynamic	None	hd	Co-transfection	[107]
		Transgenic Mouse	Adenovirus	i.v.	Therapeutic <sup>b</sup>	[25]
		Hydrodynamic	Stabilized	i.v.	Therapeutic (at 3 days)	[42]
SARS-CoV <sup>c</sup>	[108]					
Rhinovirus	[109]					
Poliovirus	[110]					
HIV <sup>d</sup>	[111]					
Papillomomavirus	[112]					
Cytomegalovirus	[113]					

hd, hydrodynamic injection; i.v., intravenous injection; i.n., intranasal administration.

<sup>a</sup>RSV, respiratory syncytial virus.

<sup>b</sup>Transgenic mice constitutively express and replicate HBV, therefore this is a model of chronic HBV, therefore time of treatment is not related to time of viral delivery.

<sup>c</sup>SARS-CoV, severe-acute respiratory syndrome-coronavirus.

<sup>d</sup>HIV, human immunodeficiency virus.

Table 3  
Therapeutic targets of RNAi tested in vivo

Field	Disease	Target	Route	Vehicle	References
Neurological	Amyotrophic lateral sclerosis	SOD1	i.m.	Lentivirus	[126]
		SOD1	Intraspinal	Lentivirus	[127]
	Spinocerebellar ataxia	Ataxin1	i.c.	AAV	[40]
	Huntington's disease	Huntingtin	i.c.	AAV	[128]
	Neuropathic pain	P2X3 cation channel	Intrathecal	None	[39]
Ocular	Inflammation in eye	TGF $\beta$ RII	Local	None	[129]
	AMD	VEGF	Local	Transit TKO	[130]
	Herpetic stromal keratitis	VEGF/R	i.v.	Ligand directed	[131]
Hearing	Autosomal dominant	Gap junction $\beta$ 2	Local	Liposome	[132]
Inflammation	Rheumatoid arthritis	TNF $\alpha$	Local	None	[133]
	Sepsis	TNF $\alpha$	i.p.	None	[44]
Apoptosis	Acute liver failure	Fas	hd	None	[134]
		Caspase 8	hd/p.v.	None	[135]
	Liver ischemia/reperfusion	Caspase 8/3	hd	10%/lipiodol	[136]
	Renal ischemia/reperfusion	Fas	hd	None	[137]
	Lung ischemia/reperfusion	Heme oxygenase1	i.n.	None	[37]
Metabolism	Obesity	AGRP	i.c.	None	[38]
	Cholesterol	ApoB	i.v.	Modified	[41]

hd, hydrodynamic injection; i.v., intravenous; i.n., intranasal; i.c., intracranial; i.m., intramuscular; AAV, adeno-associated virus; AMD, age-related macular degeneration; AGRP, agouti related peptide.

Table 4  
Anti-cancer RNAi targets tested in vivo

Target	Route	Vehicle	References
Bcl-2	i.v.	Liposome	[145]
Cxcr4	i.v.	None	[146]
Focal adhesion kinase	i.v.	None	[147]
EphA2	i.v.	None	[148]
Polo-like kinase 1	i.v.	ATA-treated	[149]
Colony-stimulating factor	i.t.	None	[150]
survivin	hd	DNA	[151]
CEACAM6	i.v.	None	[152]
EGFR	i.v.	Ligand-targeted	[49]
ErbB2/neu (HER2)R	i.p.	PEI-complex	[47]
Skp-2	i.t.	Adenovirus	[153]
Spingosine-1 phosphate-R	i.t.	Liposome	[154]
RhoA	i.t.	None	[155]
VEGF-R	i.v.	Ligand-targeted	[46]
VEGF	i.t./i.v.	Atelocollagen	[156]
FGF4	i.t.	Atelocollagen	[157]

i.v., intravenous; i.t., intratumoral; hd, hydrodynamic injection; ATA, aurintricarboxylic acid; CEACAM6, carcinoembryonic antigen-related adhesion molecule 6.

## 4. Therapeutic applications

### 4.1. Viral infection

One of the earliest proposed therapeutic uses of RNAi was to inhibit viral infection, and several companies developing RNAi-based therapies have ongoing programs focused on viral targets (Table 1).

**Targeting viral RNAs.** Many reports have shown that pre-treatment or co-treatment with virus-specific siRNAs can be used to inhibit the expression and/or replication of numerous viruses in vitro and in vivo (reviewed in [94]) (Table 2). In particular, investigations into the potential antiviral benefits of RNAi have focused heavily on HIV (reviewed in [111]) and

the hepatitis viruses (reviewed in [97,114,115]), as currently available treatment options against these viruses are inadequate. Furthermore, the availability of multiple well-defined HBV mouse models [116,117] has made this virus a popular target for in vivo proof-of-concept studies. Initial in vivo hydrodynamic transfection experiments demonstrated that simultaneous delivery of HBV expression plasmids and HBV-specific siRNAs (or shRNA expression vectors) to the mouse liver can prevent the induction of HBV gene expression and replication [106,107,118]. To expand on those studies, we and others have further examined the therapeutic potential of RNAi for the treatment of chronic HBV infection using mouse models in which ongoing viral gene expression and replication are established in the liver prior to siRNA delivery [25,42]. Although it has been shown that some viral RNAs are resistant to silencing [85–87] and some mammalian viruses, like HIV, may encode proteins that interfere with RNAi activity [7], in the case of HBV, significant inhibition of viral gene expression and replication was achieved by therapeutic administration of RNAi effectors in 2 different mouse models. Likewise, inhibition of influenza virus [35], coxsackievirus B3 [99], and respiratory syncytial virus (RSV) [36] infections have been inhibited by siRNAs delivered after the establishment of infection in mice.

**Targeting host cell genes.** Because viruses can rapidly generate escape mutations when siRNAs are targeted directly to their genome [78–82,110], another potential RNAi antiviral strategy is to inhibit the expression of cellular factors that perpetuate the infection. In particular, HIV cellular receptors, such as CD4 and CCR5, are appealing targets for inhibition as this could significantly block initial viral entry into susceptible cell types [119–123]. Before implementing this type of indirect inhibition approach, however, it would be important to determine what other effects might occur as a result of down regulating the particular host gene in ques-

tion, and this could limit the feasibility of this strategy against some viruses.

In terms of the potential for viral clearance, RNAi knock down of viral RNAs alone is unlikely to completely eliminate viral infection, however, it may still prove to be an effective antiviral if viral replication and spread can be reduced sufficiently to allow the endogenous host immune response to successfully combat the infection. For example, in the case of HBV, even chronically infected patients maintain a virus-specific cytotoxic T lymphocyte (CTL) response throughout the course of infection. Although that immune response is not strong enough to independently clear the virus, it does continue to destroy cells expressing HBV antigens meaning that any cells in which HBV has been silenced would have a survival advantage and may be selected over time to repopulate the liver.

#### 4.2. Neurological disease

Parkinson's disease, Huntington's disease (HD), fragile X syndrome, amyotrophic lateral sclerosis (ALS), and spinobulbar muscular atrophy, are just some of the prominent neurological diseases for which RNAi-based therapies might prove useful (reviewed in [124,125]) (Table 3). The germane commonality among these neurodegenerative disorders is that they result from dominant mutations in a single allele. Hence, in all cases, the sequence specificity offered by RNAi may provide a means of inhibiting expression of the mutant target gene while allowing the essential wild type allele to be expressed.

Recent examples of the ability of siRNAs to distinguish single nucleotide differences between wildtype and mutant RNAs have been demonstrated in models of ALS, which is a lethal motor neuron degenerative disease for which there is currently no treatment [138]. ALS results from single nucleotide mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1). In 2003, Ding et al. [14] reported that siRNAs and PolIII-driven shRNAs could be designed to selectively silence a dominant mutant ALS allele. More recently, two reports have confirmed the effectiveness of this approach in a transgenic SOD1(G93A) mouse model of ALS [126,127]. In both studies, lentiviral vectors were used to deliver shRNAs specific for the mutant SOD1. Both intramuscular and intrasplenic injection resulted in reduction of mutant SOD1(G93A) expression leading to a prolonged survival of motor neurons, a delay in the onset of ALS symptoms, and an increase in lifespan. Although the in vivo delivered shRNAs in these experiments were specific for the human transgene due to sequence differences between the mouse and human genes, the previous study by Ding et al., demonstrate the feasibility of distinguishing more closely related human SOD1 alleles. In terms of general clinical application however, the bigger issue may be that ALS can be caused by more than 100 distinct SOD1 mutations. To address this issue, Xia et al. [139], have tested a "replacement RNAi strategy" in which they target inhibition of all mutant and wildtype forms of SOD1, while simultaneously delivering a functional SOD1 gene that is resistant to their siRNAs due to the presence of a silent mutation. If gene therapy approaches, such as lentivirus, are developed for the delivery SOD1-specific siRNA, it should also be possible to use this type of replacement RNAi strategy.

Another class of dominant negative mutant targets is found in at least 8 progressive neurodegenerative disorders, which in-

clude HD and spinocerebellar ataxia (SCA1). These diseases are caused by polyglutamine expansions in which CAG trinucleotide repeats are found in the mutant allele. In disease models of both HD and SCA1 the mutant protein has been shown to be toxic to neurons and that repression of the mutant gene is clinically beneficial in reversing motor dysfunction [140–142]. Two recent reports have used AAV vectors to express disease-specific shRNAs in the brain of transgenic mice that model HD [128] and SCA1 [40]. In both cases, expression of the mutant gene was reduced resulting in an improvement in behavior and neuropathological abnormalities. One caveat the authors acknowledge in both studies is that due to the nature of the transgenic model, the in vivo delivered shRNAs were by default specific for the mutant human transgene based on sequence differences between the mouse and human genes. However once again, previous in vitro studies have demonstrated that allele-specific targeting of genes containing polyglutamine expansions is possible [15]. Interestingly however, while it was determined that siRNA targets within the CAG repeat region do not necessarily result in allele-specific inhibition, the authors demonstrated that siRNAs could be designed to distinguish these alleles based on associated single-nucleotide polymorphisms (SNPs), which may or may not functionally contribute to the disease phenotype [15].

In addition to targeting dominant mutant alleles, RNAi-based approaches could also prove useful for the treatment of other forms of neurological disease in which inhibition of specific processing/regulatory pathways is required. For example, Alzheimer's is caused by an increase in  $\beta$ -amyloid production, which requires cleavage by  $\beta$ -secretase (BACE1), an enzyme that is up regulated in the brain of Alzheimer's patients. Hence, siRNAs targeting the downregulation of BACE1 may be able to halt the progression of Alzheimer's. As proof of concept, Kao et al. have used RNAi to inhibit BACE1 expression in primary mouse cortical neurons and observed the expected decrease in  $\beta$ -amyloid production [143]. Similarly, as more is learned about the molecular mechanisms that mediate neurotoxicity in acute adverse neurological events, such as stroke and spinal cord injury, it is likely that targets can be identified for minimizing the events that lead to neuron damage and facilitating restorative processes.

#### 4.3. Oncology

While conventional chemotherapy has proven to be an effective means of killing cancerous cells, it lacks the selectivity required to distinguish tumor cells from normal cells resulting in a significant degree of collateral cell death. Therefore, a therapy that could be targeted specifically to cancer cells would provide a more attractive treatment option. While efficient delivery and targeting are ongoing issues for the development of RNAi-based drugs, the potential utility of RNAi in inhibiting cancer cell proliferation has recently been demonstrated in many in vitro as well as in vivo studies (Table 4) (reviewed in [144]).

*Direct targeting of oncogenes.* Again, the ability of siRNAs to discriminate between mutant and wild type alleles presents the same advantages in the field of oncology as it does for the dominant mutant neurodegenerative disorders discussed above. Multiple oncogenic gene fusions formed by chromosomal translocation in leukemia and lymphoma have been readily inhibited by RNAi approaches in vitro (reviewed in

[144]), while more stringent allele-specific targeting has been achieved for several single nucleotide mutant forms of ras and p53, which are both prevalent in human cancers [12,89,158,159].

**Drug sensitization.** Although direct inhibition of an oncogene may slow tumor growth, it does not eliminate the cancerous cells. Furthermore, reducing the rate of tumor cell proliferation might be expected to reduce the effectiveness of traditional therapies that preferentially target actively dividing cells. Hence, the potential use of RNAi to facilitate the effects of traditional cancer therapeutics has been proposed. For example, targeted suppression of the anti-apoptotic *bcl-2* gene sensitizes cells to chemotherapy agents, such as etoposide and daunorubicin [158,160,161]. Likewise, siRNA silencing of double-strand break repair proteins, such as the DNA-dependent protein kinase catalytic subunit, confer enhanced radio and chemosensitivity to tumor cells [162,163]. RNAi might also complement chemotherapy in the treatment of patients that have developed multidrug resistance. While over 30% of cancer patients develop resistance to chemotherapeutic agents due to an over expression of the multidrug resistance gene (MDR1) [164], RNAi-mediated suppression of MDR1 has been shown to re-sensitize cells to the effects of chemotherapy [165,166]. Hence, if RNAi effectors could be specifically delivered to tumors, these cells could be made preferentially sensitive to traditional cancer therapies.

**Inhibition of invasion and migration.** Finally, another cancer treatment approach being investigated is the use of RNAi to indirectly control tumor growth by inhibition of processes required for tumor spread. For example, in the case of breast cancer, transformed cells express the chemokine receptor CXCR4, which promotes metastasis to organs abundant in CXCR4 ligand [167]. Several groups have shown RNAi-mediated inhibition of CXCR4 results in reduced cell invasion *in vitro*, as well as a block in breast cancer metastasis in xenograph animal models [146,167,168]. As a more general approach, members of the vascular endothelial growth factor (VEGF) family, which are involved in the generation of new blood vessels required for tumor growth, are being tested as a potential anti-cancer treatment targets (reviewed in [169]). After transplanting tumor cells subcutaneously into mice, two groups have shown that intratumoral injections of VEGF-specific siRNAs, either naked or complexed with atelocollagen, blocked angiogenesis and limited tumor growth [156,170]. More recently, Schiffelers et al. [46] achieved similar results delivering VEGF receptor-specific siRNAs to xenograph tumors by intravenous injection of ligand-targeted, stabilized nanoparticles.

#### 4.4. Ocular disease

Angiogenesis additionally has a causal role in several ocular diseases including age-related macular degeneration (AMD), herpetic stromal keratitis, and diabetic retinopathy [171]. VEGF has been found to be directly involved in the destructive vascularization associated with these ocular disease. The ability of VEGF-specific siRNAs to reduce VEGF-dependent vascular invasion of the eye has not only been demonstrated in animal models [130,131] (Table 3), but phase I AMD clinical trials in patients is ongoing as well [34]. Notably, however, there is a competing technology in this area, as an anti-VEGF aptamer therapeutic, Pegaptanib

sodium, was approved for the treatment of AMD in December 2004 [172].

#### 4.5. Inflammation and apoptosis

In some diseases, the pathology observed is caused by the activation of innate cellular processes. Hence, by targeting key molecules involved in these pathways, RNAi therapeutics may provide a means of controlling the cellular processes responsible. For example, tumor necrosis factor (TNF) $\alpha$  is a proinflammatory cytokine involved in the chronic pathogenesis of rheumatoid arthritis (RA). While drugs currently being used to block the action of TNF $\alpha$  have been shown to be effective in reducing inflammation and slowing RA progression, several risks, such as congestive heart failure, demyelinating diseases, systemic lupus erythematosus, lymphoma, and serious infections, have been associated with the use of systemic TNF $\alpha$ -blockers [173]. In a preclinical mouse model of collagen induced arthritis, Schiffelers and colleagues recently demonstrated that local injection and electroporation of TNF $\alpha$ -specific siRNAs effectively inhibited paw inflammation in mice [133]. Furthermore, using an intravenously injected luciferase reporter, the authors subsequently showed that joint injection and electroporation of luciferase-specific siRNAs only inhibited the reporter gene locally. These studies suggest that in lieu of conventional anti-TNF $\alpha$  blockers, local delivery of TNF $\alpha$ -specific siRNAs might provide a safer means of reducing inflammation in RA patients.

While many proposed RNAi therapeutic targets are related to chronic and/or progressive diseases, it is important to note that RNAi could also potentially serve a valuable function in limiting acute responses as well. In a mouse model of sepsis, it has been shown that pretreatment with TNF $\alpha$ -specific siRNAs injected intraperitoneally was able to increase survival rate sixfold after lethal lipopolysaccharide challenge [44]. Similarly, one of the first experiments to demonstrate the therapeutic potential of RNAi used fas-specific siRNAs to protect mice against fulminant hepatitis [134], and another study reported that caspase 8-specific siRNA treatment also prevented acute liver failure [135]. These studies illustrate the concept that siRNAs might be used effectively to control acute responses whether that means limiting dangerous, uncontrolled inflammation or preventing hepatocyte apoptosis and preserving liver function (Table 3).

## 5. Conclusion

Although not complete, the above list of potential therapeutic applications of RNAi is intended to illustrate how the advantages inherent in RNAi technology (*Specificity*, *Potency*, and *Versatility*) could possibly translate into significant progress in molecular medicine. However, it is important to also revisit the sobering challenges that face all sequence-specific gene silencing technologies (*Delivery*, *Safety*, and *Efficacy*) before making any predictions about the future of this promising, yet immature, technology. Fortunately, the development of RNAi technology has been facilitated by the many years of previous research into antisense drug delivery and the enormous amount of genetic information recently obtained from the Human Genome Project. With the convergence of all these

events, momentum alone seems to be providing the extra push that could make a leap forward possible.

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