REVIEWS

Strategies for silencing human disease using RNA interference

Daniel H. Kim and John J. Rossi

Abstract | Since the first description of RNA interference (RNAi) in animals less than a decade ago, there has been rapid progress towards its use as a therapeutic modality against human diseases. Advances in our understanding of the mechanisms of RNAi and studies of RNAi *in vivo* indicate that RNAi-based therapies might soon provide a powerful new arsenal against pathogens and diseases for which treatment options are currently limited. Recent findings have highlighted both promise and challenges in using RNAi for therapeutic applications. Design and delivery strategies for RNAi effector molecules must be carefully considered to address safety concerns and to ensure effective, successful treatment of human diseases.

Short hairpin RNAs
(shRNAs). A class of small RNAs with a stem of 19–29 base pairs and a loop of 4–10 nucleotides that are processed by Dicer into small interfering RNAs. shRNAs are expressed from vectors to induce RNAi.

Interferons
A class of glycoproteins that are upregulated in response to exogenous ssRNA or dsRNA as a cellular defence mechanism against RNA viral infection.

Division of Molecular Biology, Graduate School of Biological Sciences, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, California 91010, USA.
Correspondence to J.J.R.
e-mail: jrossi@bricoh.edu
doi:10.1038/nrg2006

The realm of RNA interference (RNAi) has expanded at a remarkable rate since the initial characterization of RNAi in the nematode *Caenorhabditis elegans*[1]. Soon after this, RNAi was shown to occur in mammalian cells in response to double-stranded small interfering RNAs (siRNAs) of ~21 nt in length[2] that serve as the effector molecules of sequence-specific gene silencing. Mechanistic insights followed rapidly during the ensuing years, and with them came the increasing hope that RNAi pathways could be harnessed for the therapeutic intervention of human diseases[3]. The key therapeutic advantage of using RNAi lies in its ability to specifically and potently knock down the expression of disease-causing genes of known sequence. Furthermore, the relatively short turnaround time for efficacy testing of potential therapeutic RNAi molecules, and the fact that even newly discovered pathogens are theoretically amenable to rapid targeting, has caused great excitement about the potential of RNAi for treating a wide range of diseases.

Recent findings have highlighted the effectiveness of RNAi in therapeutically relevant settings, the results of which have spurred cautious optimism about the promise of RNAi-based therapies. The first clinical applications of RNAi have been directed at the treatment of wet, age-related macular degeneration (AMD)[4,5] and respiratory syncytial virus (RSV) infection[6]. Therapies based on RNAi are also in preclinical development for other viral diseases[7,8], neurodegenerative disorders[9] and cancers[10], although a number of challenges need to be addressed and improvements made for RNAi-based therapies to realize their full potential. A progressively more detailed understanding of the basic mechanisms of RNAi has been important in developing diverse RNAi effector molecules with improved levels of potency and efficacy. For example, synthetic siRNAs and expressed short hairpin RNAs (shRNAs)[11] both have specific advantages and disadvantages, which are important considerations when designing RNAi-based therapies for a particular disease. In addition, although many *in vivo* studies have shown the potential effectiveness of various RNAi-based strategies, other studies have highlighted challenges that arise as a result of using an endogenous cellular mechanism for therapeutic benefit. Unwanted side effects have included induction of type 1 interferon (IFN) responses[12] and saturation of endogenous RNAi pathway components[13], indicating that caution is necessary when designing effector molecules for delivery into target cells. The issue of cell-specific or tissue-specific delivery is another key challenge in developing RNAi-based therapies. Various strategies for non-viral and viral delivery of RNAi triggers have recently been shown to be effective in disease models, raising the hope that clinical studies of RNAi-based therapies will be extended to an increasing list of diseases in the near future[14].

Here we provide an overview of the current mechanistic understanding of RNAi, leading into a discussion of how potency can be maximized and off-target effects (OTEs) minimized or avoided in therapeutic applications. We then discuss strategies that have been investigated for effective delivery *in vivo*. Finally, we conclude with an overview of the therapeutic applications of RNAi currently in development and the outlook for the wider use of RNAi-based therapies in the future.

Mechanisms of RNAi-mediated gene silencing

RNAi pathways are guided by small RNAs that include siRNAs and microRNAs (miRNAs), which derive from imperfectly paired hairpin RNA structures naturally encoded in the genome[15]. RNAi effector molecules induce gene silencing in several ways: they direct sequence-specific cleavage of perfectly complementary mRNAs and translational repression and transcript degradation for imperfectly complementary targets. RNAi pathways can also direct transcriptional gene silencing (TGS) in the nucleus[16,17], although mechanistic details of TGS are not yet well established in mammalian systems (FIG. 1).
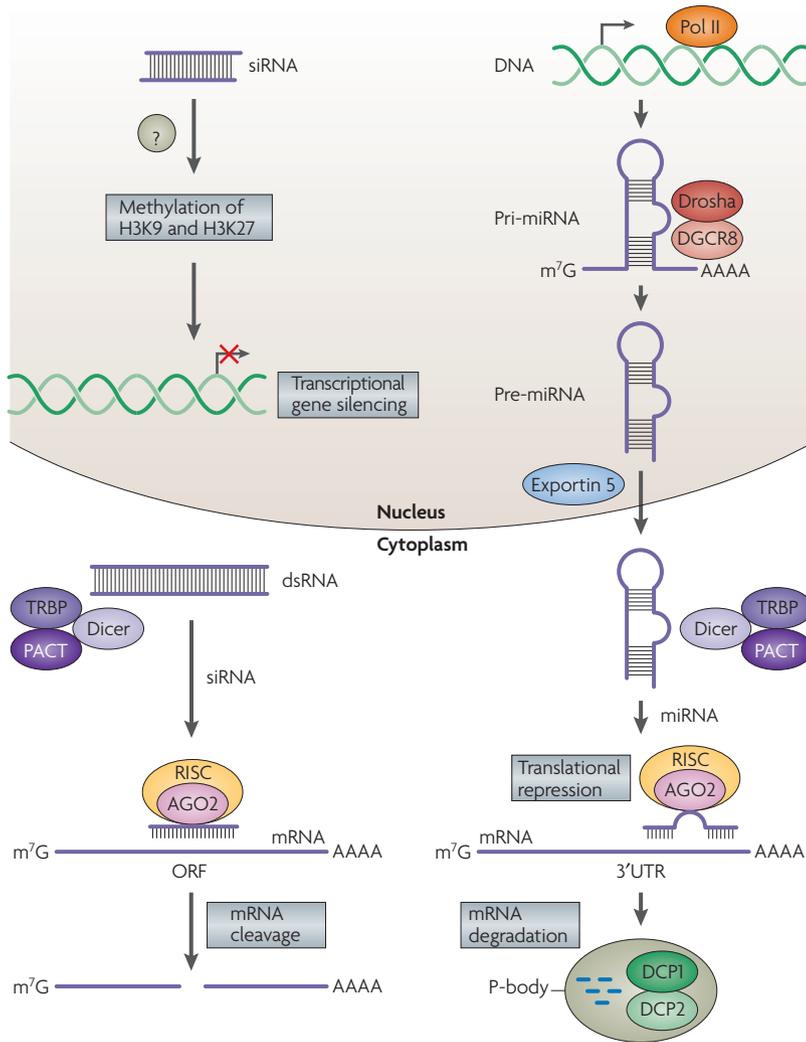
Post-transcriptional gene silencing by siRNAs. Exogenous siRNAs target complementary mRNAs for transcript cleavage and degradation in a process known as post-transcriptional gene silencing (PTGS)[18]. In nematodes, insects and plants, this pathway functions as an innate antiviral defence mechanism, in which viral double-stranded RNA (dsRNA) molecules are processed by the RNase III enzyme Dicer[19] into siRNAs that mediate the RNAi response. Whether or not siRNA-mediated PTGS exists in mammalian cells for intrinsic immunity against viral infections is unclear, and remains an area for further investigation.

Effective PTGS requires perfect or near-perfect Watson–Crick base pairing between the mRNA transcript and the antisense or guide strand of the siRNA, and results in cleavage of the mRNA by the RNA-induced silencing complex (RISC)[20]. The endonuclease Argonaute 2 (AGO2) is responsible for the cleavage mechanism of RISC, and AGO2 is the only member of the Argonaute subfamily of proteins with observed catalytic activity in mammalian cells[21]. RISC activation is initially thought to involve AGO2-mediated cleavage of the sense or passenger strand of the double-stranded siRNA[22,23], generating the single-stranded antisense strand that serves to guide RISC to complementary sequences in target mRNAs (FIG. 2). This guide strand is bound within the catalytic, RNase H-like PIWI domain of AGO2 at the 5′ end[24] and a PIWI–Argonaute–Zwille (PAZ) domain that recognizes the siRNA 3′ end[25].

The cleavage of targeted mRNA takes place between bases 10 and 11 relative to the 5′ end of the siRNA guide strand[26], leading to subsequent degradation of the cleaved mRNA transcript by cellular exonucleases[27]. On activation by the siRNA guide strand, RISC can undergo multiple rounds of mRNA cleavage to mediate a robust PTGS response against the target gene[28]. PTGS by mRNA cleavage has been exploited as the method of choice for potential therapeutic applications of RNAi because of the potency of this catalytic gene-silencing pathway.

The microRNA pathway. The endogenous miRNA pathway serves as a cellular rheostat for fine-tuning gene expression during development and differentiation[29]. The 3′ untranslated regions (3′ UTRs) of mRNAs are targeted by miRNAs with which they share partial sequence complementarity[30,31]. These endogenous small RNAs of ~22 nt in length induce PTGS through translational repression. This is often accompanied by subsequent mRNA degradation, which occurs in cytoplasmic compartments known as processing bodies (P-bodies)[32]. When an miRNA has complete sequence complementarity with a target mRNA, it instead directs cleavage of the mRNA transcript through RISC activity. One such example, miR-196-directed cleavage of *Hoxb8*

Figure 1 | **Mechanisms of RNA interference in mammalian cells.** As shown in the pathway at the bottom left, cytoplasmic double-stranded RNAs (dsRNAs) are processed by a complex consisting of Dicer, TAR RNA-binding protein (TRBP) and protein activator of protein kinase PKR (PACT) into small interfering RNAs (siRNAs), which are loaded into Argonaute 2 (AGO2) and the RNA-induced silencing complex (RISC). The siRNA guide strand recognizes target sites to direct mRNA cleavage, which is carried out by the catalytic domain of AGO2. siRNAs complementary to promoter regions direct transcriptional gene silencing in the nucleus through chromatin changes involving histone methylation (top left); the precise molecular details of this pathway in mammalian cells are currently unclear. As shown in the pathway on the right, endogenously encoded primary microRNA transcripts (pri-miRNAs) are transcribed by RNA polymerase II (Pol II) and initially processed by Drosha–DGCR8 (DiGeorge syndrome critical region gene 8) to generate precursor miRNAs (pre-miRNAs). These precursors are exported to the cytoplasm by exportin 5 and subsequently bind to the Dicer–TRBP–PACT complex, which processes the pre-miRNA for loading into AGO2 and RISC. The mature miRNA recognizes target sites in the 3′ untranslated region (3′ UTR) of mRNAs to direct translational inhibition and mRNA degradation in processing (P)-bodies that contain the decapping enzymes DCP1 and DCP2. H3K9, histone 3 lysine 9; H3K27, histone 3 lysine 27; m7G, 7-methylguanylate; ORF, open reading frame.
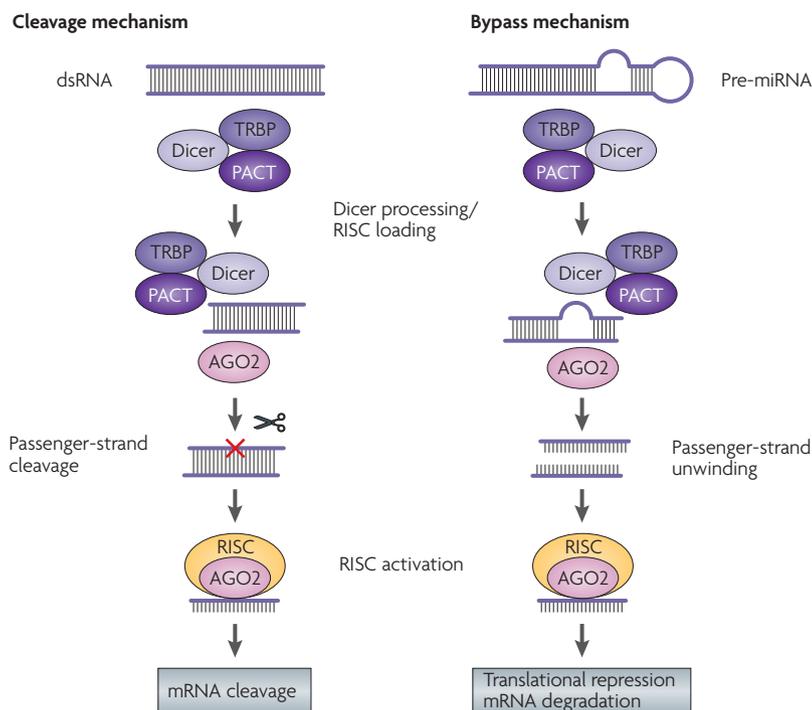
Cleavage mechanism

Bypass mechanism

Figure 2 | **RISC loading and activation.** Double-stranded RNAs (dsRNAs) and precursor microRNAs (pre-miRNAs) are processed by a complex comprising Dicer, TAR RNA-binding protein (TRBP) and protein activator of protein kinase PKR (PACT), facilitating loading of the small interfering RNA (siRNA) or microRNA (miRNA) duplex into Argonaute 2 (AGO2) and RNA-induced silencing complex (RISC). When the RNA duplex loaded into RISC has perfect sequence complementarity, AGO2 cleaves the passenger strand so that active RISC is produced that contains the guide strand, which is complementary to the target sequence. When the RNA duplex loaded into RISC has imperfect sequence complementarity a bypass mechanism is used, in which a helicase activity is required to unwind the passenger strand from the guide strand and to generate the mature miRNA strand, producing active RISC.

Processing bodies
Cytoplasmic bodies that contain enzymes involved in mRNA turnover, such as the decapping enzymes DCP1 and DCP2, and sequester mRNAs from the translational machinery.

(homeobox 8), has been shown to occur in mammalian cells[33], illustrating a level of functional overlap between siRNA and miRNA-directed gene-silencing pathways.

Long primary miRNA transcripts (pri-miRNAs) are generally transcribed by RNA polymerase II (Pol II) (REF. 34) in the nucleus (although a recent finding also describes miRNAs transcribed by RNA Pol III (REF. 35)) and are processed by the RNase III enzyme Drosha into ~70 nt stem-loop structures known as precursor miRNAs (pre-miRNAs)[36]. Drosha functions with the dsRNA-binding protein of DiGeorge syndrome critical region gene 8 (DGCR8) in a complex known as the microprocessor to generate these pre-miRNAs[37]. The dsRNA-binding protein exportin 5 then transports the pre-miRNA into the cytoplasm in a Ran-GTP-dependent manner[38,39], where Dicer and its dsRNA-binding protein partners, HIV-1 TAR RNA-binding protein (TRBP)[40] and protein activator of protein kinase PKR (PACT)[41], process the pre-miRNA and load the ~22 nt mature miRNA into RISC[42] (FIG. 1). The miRNA-loading pathway into RISC does not seem to involve cleavage of the miRNA passenger strand, and might instead use a bypass mechanism that requires helicase activity to unwind and discard the passenger strand; imperfect

sequence homology between the mature miRNA strand and its complementary passenger strand might prevent AGO2 from cleaving the passenger strand[42]. Once the passenger strand has been unwound or discarded and the mature miRNA binds to its target mRNA 3'UTR, RISC directs translational repression and subsequent mRNA degradation to silence gene expression (FIG. 2). The seed sequence of a mature miRNA, which encompasses the first 2–7 or 2–8 nucleotides from its 5'end[43], must have complete complementarity with its target, whereas mismatched nucleotides in the 3' end of the miRNA strand are more tolerated. Although the effector stages of this endogenous pathway have not been used for therapeutic development, perfect duplex siRNA sequences have been introduced into pri-miRNA and pre-miRNA backbones, generating miRNA mimics that are processed by the miRNA pathway but trigger the more potent PTGS pathway of mRNA cleavage once loaded into RISC[44].

Transcriptional gene silencing by siRNAs. Silencing of gene expression at the transcriptional level was first shown to take place in the nuclei of plant and fungal cells. TGS regulates gene expression through changes in chromatin mediated by siRNAs and the RNAi machinery[16,17] (FIG. 1). In mammalian cells, some level of TGS and histone methylation has been shown to occur in response to exogenous, promoter-targeting siRNAs[45–49], although the precise mechanism by which this is achieved is poorly understood. TGS might potentially be used in future therapeutic applications of RNAi for prolonged, epigenetic gene silencing[50], but no such applications have been tested in preclinical models so far.

Designing potent triggers of RNAi

Delivered siRNAs. Most of the proposed clinical applications of RNAi incorporate chemically synthesized 21-nt siRNA duplexes that have 2-nt 3' overhangs (FIG. 3a), allowing large-scale synthesis and uniform production of siRNA molecules that are also amenable to chemical modifications that increase their stability. Knockdown of gene expression is accomplished by designing siRNA sequences that target the coding and non-coding regions of mRNAs with perfect complementarity to induce PTGS. Several commercial entities involved in the manufacturing of siRNAs provide effective design algorithms online, which are based on a combination of mRNA target sequence and secondary structures, siRNA duplex end-stabilities, and aim to minimize potential sequence-dependent OTEs.

Longer siRNAs (27mers) and shRNAs (29 nt) that are chemically synthesized serve as substrates for Dicer processing (FIG. 3a), and for some siRNA–target combinations the use of these longer dsRNAs can increase the potency of PTGS[51,52]. Dicer and TRBP–PACT might comprise a loading platform for RISC formation[40–42], and incorporating this loading step in the RNAi pathway through the use of Dicer substrates elicits a more potent gene-silencing effect. 27mers are designed so that they are asymmetrical, with one 2-nt 3' overhang and one blunt end. Because Dicer recognizes the 2-nt 3' overhang for processing, this design ensures that a

a Synthetic siRNAs

Unmodified siRNA
(21 nt)

2′-O-Methylpurines — Me Me

2′-Fluoropyrimidines — F F

Chemically modified siRNA

D D DNA bases

Asymmetrical Dicer–substrate siRNA (27 nt)

Synthetic Dicer–substrate shRNA (29 nt)

b Expressed shRNAs

Pol III

Pol III

Pol II or Pol III

G:U wobble

shRNA lhRNA

3 shRNAs
(3 promoters)

Polycistronic shRNAs
(1 promoter)

miRNA
mimic shRNA

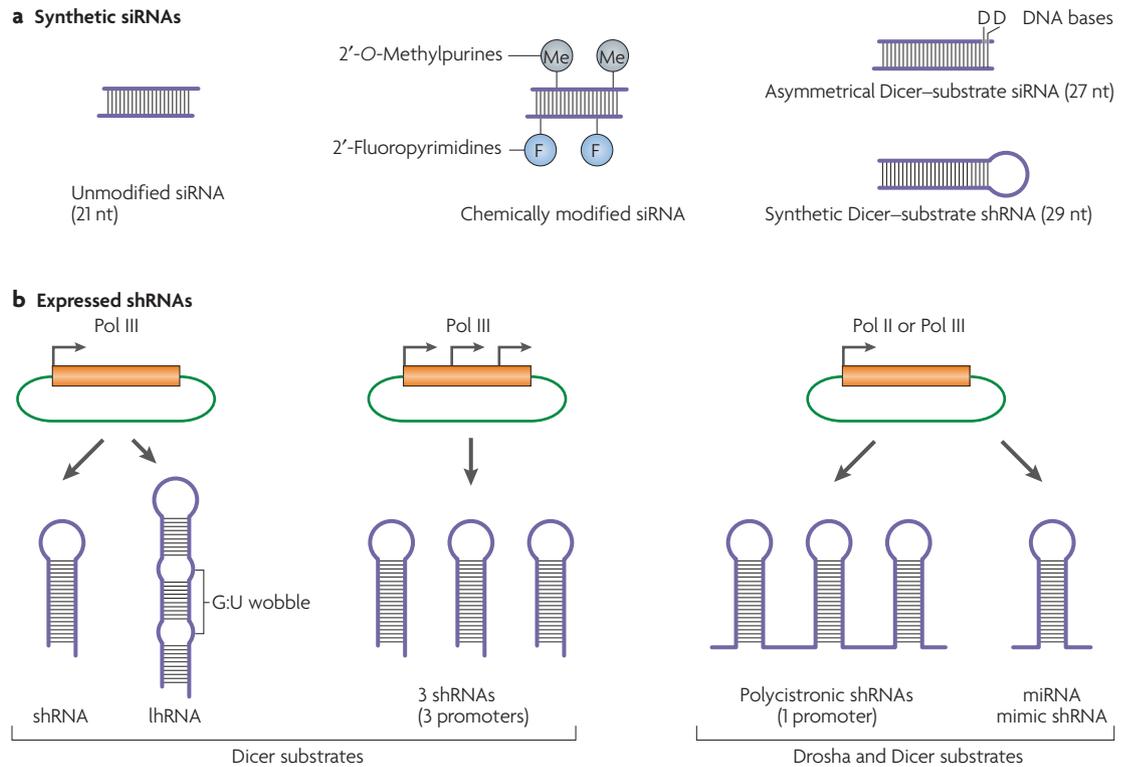Dicer substrates

Drosha and Dicer substrates

Figure 3 | **RNA interference effector molecules. a** | Synthetic small interfering RNAs (siRNAs; left panel) are administered *in vivo*. These can be produced with chemical modifications (middle panel) — such as 2′-O-methylpurines or 2′-fluoropyrimidines — which can be added to increase stability. Asymmetrical Dicer–substrate siRNAs can also be produced (right panel). These have a blunt end that includes two DNA bases (D), whereas the other end has a 2-nt 3′ overhang. This ensures that a single species of siRNA is generated by Dicer, which processes the blunt end. Longer synthetic short hairpin RNAs (shRNAs) are also processed as Dicer substrates. **b** | Expression vectors drive high levels of shRNA expression from polymerase III (Pol III) promoters (left panel). Long hairpin RNAs (lhRNAs) generate multiple Dicer-processed siRNA species, suggesting mammalian Dicer is processive (M. Sano and J.J.R., unpublished observations). Multiple separate Pol III promoters can be used in one vector to drive expression of several different shRNAs (middle panel). Vectors carrying Pol II or Pol III promoters generate longer precursor RNAs, including polycistronic shRNA transcripts and microRNA (miRNA) mimics that are processed by both Drosha and Dicer (right panel).

single siRNA product is produced. However, the blunt end, which includes DNA bases[11], might trigger low levels of interferon induction[53] (see below), but the lower concentrations of 27mers required to silence gene expression might avoid or minimize such an interferon response.

Expressed shRNAs. siRNAs transiently silence gene expression, because their intracellular concentrations are diluted over the course of successive cell divisions. By contrast, expressed shRNAs mediate long-term, stable knockdown of their target transcripts for as long as transcription of the shRNAs takes place[54,55] (FIG. 3b). RNA Pol II and III promoters are used to drive expression of shRNA constructs[11], depending on the type of expression required. Consistent with their normal cellular roles in producing abundant, endogenous small RNAs, Pol III promoters (such as U6 or H1) drive high levels of constitutive shRNA expression, and their transcription initiation points and termination signals (4–6 thymidines) are well defined. Pol II promoter-driven shRNAs can be expressed tissue-specifically and are transcribed as longer precursors that mimic pri-miRNAs and have cap

and polyA signals that must be processed[56]. Such artificial miRNAs/shRNAs are efficiently incorporated into RISC, contributing to a more potent inhibition of target-gene expression[44]; this allows lower levels of shRNA expression and might prevent saturation of components in the RNAi pathway. An additional advantage of Pol II promoters is that a single transcript can simultaneously express several miRNA and mimic shRNAs[57] (FIG. 3b). This multiplexing strategy can be used to simultaneously knock down the expression of two or more therapeutic targets, or to target several sites in a single gene product.

Avoiding harmful side effects
Seed-sequence-dependent off-target effects. The siRNA guide strand might function as a miRNA when it contains seed-sequence matches to mRNA 3′ UTR regions, which might lead to harmful OTEs[58] through translational repression or mRNA degradation. Such OTEs do not seem to occur if the guide-strand seed pairs in the open reading frame (ORF) of mRNA transcripts[59]; avoiding siRNA seed matches with mRNA 3′ UTRs using online 3′ UTR search algorithms would potentially

Polycistronic
A single RNA molecule that can generate several products. For miRNAs, a single polycistronic transcript contains multiple stem-loop structures encoding separate miRNAs.
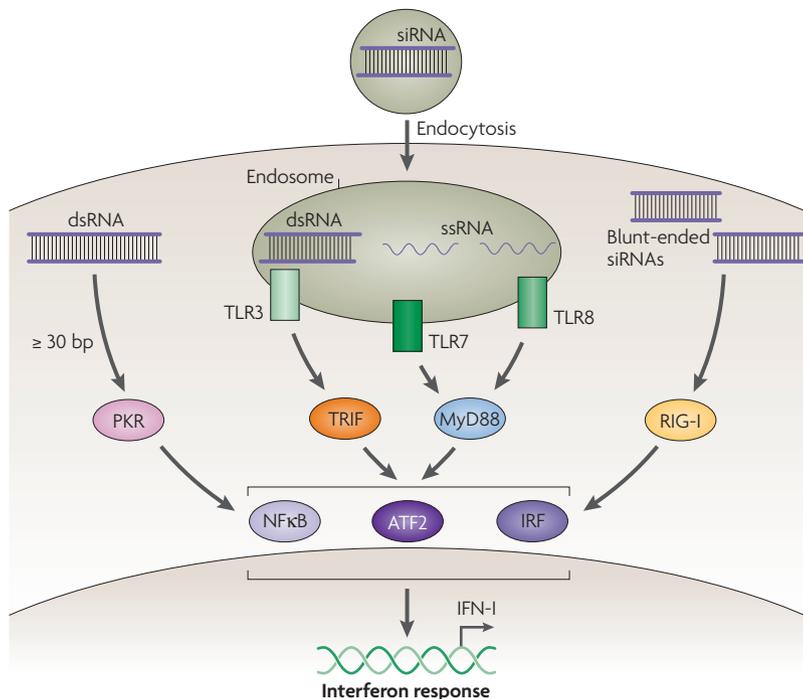
Figure 4 | **Immunostimulatory effects of RNA interference.** Double-stranded RNAs (dsRNAs) longer than 30 base pairs activate protein kinase R (PKR) and induce an interferon (IFN) response, as do blunt-ended, small interfering RNAs (siRNAs), which are detected by RIG-I. siRNAs that are incorporated into endosomes can activate Toll-like receptors (TLRs), depending on the presence of immunostimulatory motifs, inducing a type I interferon response by activating the transcription factors nuclear factor-κB (NFκB), activating transcription factor 2 (ATF2) and interferon regulatory factor (IRF). MyD88, myeloid differentiation primary response gene 88; TRIF, TLR-signalling adaptor protein.

Plasmacytoid dendritic cells
Cells of the immune system that recognize foreign pathogens through Toll-like receptors and other pattern-recognition receptors.

Endosome
A vesicle formed during the incorporation of extracellular material by endocytosis. Toll-like receptors are found in endosomal compartments.

Nanoparticle
Nanometre-scale particles that are formulated from polymers or phospholipids and are used as delivery vehicles for therapeutic applications.

reduce — but not necessarily eliminate — detrimental OTEs. Consequently, careful preclinical studies of cell viability and function should be performed when developing any RNAi-based therapies. So far, no occurrences of seed-sequence-mediated OTEs have been reported in preclinical or early clinical trials of siRNAs.

Eluding interferon responses. For RNAi to be used safely in a clinical setting, the activation of innate cellular immunity due to the introduction of foreign agents needs to be avoided. In particular, important advances have recently been made in understanding the mechanism of the interferon response to foreign pathogens and nucleic acids, providing important considerations of siRNA design to avoid triggering this pathway. Type I interferons are activated in plasmacytoid dendritic cells when specific immunostimulatory sequence motifs are present in exogenous siRNAs[60]. Previous studies had suggested that only dsRNAs greater than 30 base pairs in length could elicit an interferon response, generated through the activation of the kinase PKR and the shutting down of global gene expression[61]. However, Toll-like receptors (TLRs) expressed in endosomes have been shown to recognize single-stranded RNAs (ssRNAs) and dsRNAs, eliciting an interferon response by triggering translocation of the transcription factors nuclear factor-κB (NFκB), interferon regulatory factor

(IRF) and activating transcription factor 2 (ATF2) into the nucleus[62]. TLR3, which recognizes dsRNAs, and TLR7 and TLR8, which recognize ssRNAs, mediate interferon responses both *in vivo* and in cultured mammalian cells by recognizing endosomally incorporated siRNAs. These TLRs serve as pattern-recognition sensors of specific immunostimulatory sequence motifs that should be avoided in siRNA design (that is, 5′-GUCCUUCAA-3′ and 5′-UGUGU-3′)[60,63].

Chemically synthesized siRNAs are preferable to siRNAs transcribed by the bacteriophage T7 RNA polymerase, which leaves behind 5′ triphosphates that induce type I IFN production[64]. Blunt-ended siRNAs lacking 2-nt 3′ overhangs characteristic of Dicer processing are also immunostimulatory and are recognized by the retinoic acid gene 1 encoded RIG-I helicase[53]. The cytoplasmic RIG-I (also known as DDX58) response is distinct from the interferon response mediated by TLRs, which requires siRNA delivery through the endosomal pathway (FIG. 4).

Interferon responses can be avoided in certain cell types by using expressed shRNAs. CD34+ progenitor cell-derived plasmacytoid dendritic cells show an immune response when transfected with immunostimulatory siRNAs by lipid delivery, which requires endosomal incorporation, but identical sequences fail to induce type I IFN when expressed as shRNAs from a Pol III promoter in the context of an integrated lentiviral vector[65]. The avoidance of an immune response that is achieved using expressed shRNAs might stem from their processing by the endogenous RNAi pathway, therefore avoiding interactions with TLRs and detection by RIG-I surveillance.

Preventing saturation of endogenous silencing pathways. Although a considerable number of studies have reported the specific silencing of target genes by expressed shRNAs without detrimental effects on cell viability, several recent studies have shown that abundant shRNA expression can be toxic. In one study, high-level expression of shRNAs using the U6 promoter in adeno-associated virus (AAV) vectors caused saturation of exportin 5 and subsequent inhibition of endogenous pre-miRNA nuclear export, which proved to be lethal in 23 out of 49 mice[13]. Administering lower doses of shRNA-expressing AAV vectors was non-lethal and led to effective and prolonged therapeutic intervention against hepatitis B virus (HBV). Another study in human primary lymphocytes demonstrated that U6 promoter-driven shRNAs induced cytotoxicity, whereas using a weaker H1 promoter for expression of the same shRNAs seemed to abrogate the toxic effect[66]. These findings illustrate that optimization of shRNA expression levels is an important consideration in therapeutic settings.

Non-viral delivery of RNAi-inducing agents
Cell-specific delivery of siRNAs *in vivo* is perhaps the most important consideration in developing an effective, RNAi-based therapeutic agent. Duplex siRNAs are negatively charged polymers and so cannot easily penetrate hydrophobic cellular membranes without assisting carriers such as liposomes or nanoparticles. Unmodified

and unprotected siRNAs are also rapidly degraded by serum RNases, and stabilizing chemical modifications can be used to increase their half-life and functionality *in vivo*. Non-viral delivery methods (TABLE 1) include non-selective and cell-specific systemic delivery, which might alleviate potentially adverse side effects stemming from unwanted delivery to non-targeted cells.

Chemical modifications to siRNAs increase stability. Chemical modifications at the 2′ position of the ribose, including 2′-O-methylpurines and 2′-fluoropyrimidines, increase siRNA stability by providing resistance to RNase activity in serum[67] (FIG. 3a). 2′-O-methylmodified siRNAs have been shown to provide greater protection against *in vivo* infection with HBV when compared with analogous, unmodified siRNAs[68]. The strategic placement of these modifications is crucial, as modifications on the 5′ end of the guide strand can adversely affect silencing potency[69]. 2′-O-methyl modifications that are staggered between the two siRNA strands seem to provide an optimal balance between retention of RNAi potency and protection against degradation[67]. An additional benefit of using siRNAs with 2′-O-methyl modifications is their avoidance of interferon induction, as described above[70].

Non-selective systemic delivery of siRNAs. One effective method of systemic siRNA delivery involves intravenous injection of chemically modified siRNAs either conjugated to a cholesterol group or packaged into a protective liposomal particle. This non-selective form of systemic delivery is appropriate for certain tissue types, such as the liver and jejunum, but not for delivery to most other specific cell types. Cholesterol groups linked chemically to the 3′ hydroxyl group of the siRNA passenger strand facilitate cellular siRNA uptake through receptor-mediated endocytosis (FIG. 5a). This approach was used successfully in the delivery of siRNAs into the liver and jejunum after systemic administration in mice in a study that used siRNAs targeted against apolipoprotein B (APOB) to modify cholesterol metabolism[71]. A more than 50% reduction in *ApoB* mRNA was achieved

in the liver, and a 70% reduction in the jejunum, along with a lowering of overall cholesterol levels, providing an important proof-of-concept that systemically delivered siRNAs can be delivered to tissues by conjugation to specific molecules that promote cellular uptake.

Chemically stabilized siRNAs encapsulated in specialized lipid bilayers known as stable nucleic acid–lipid particles (SNALPs) have also been used to achieve systemic delivery of RNAi[70]. The SNALP bilayer consists of cationic and neutral lipids, with an outer coating of hydrophilic polyethylene glycol (PEG) (FIG. 5a). An important study of systemic siRNA delivery using SNALPs demonstrated an effective therapeutic RNAi response in non-human primates[72]. In cynomolgus monkeys, one dose of SNALP-mediated siRNA delivery could lower APOB, low-density lipoprotein and cholesterol levels for 11 days or more, with less than 10% of normal levels of *APOB* mRNA remaining in the liver. The silencing effect was even more potent than that observed in the studies in mice described above[70] and, importantly, no toxicities were observed. Although it remains unclear whether OTEs could arise from nonspecific siRNA delivery to other tissues and/or cell types over repeated administrations, the SNALP delivery method has been shown to be effective *in vivo* for therapeutic targets expressed in the liver.

Selective systemic delivery of siRNAs. A practical consideration for therapeutic RNAi is the dosage of siRNAs that must be administered for *in vivo* efficacy. One disadvantage of non-selective systemic delivery stems from the large quantities of siRNAs required for gene silencing to occur *in vivo*. Systemic delivery with selective targeting of siRNAs to specific cell-surface receptors is advantageous from the standpoint of lower dosage requirements and reduction of potential OTEs in non-target tissues. Therapeutic siRNAs have been coupled to antibody fragments and aptamers or packaged into nanoparticles coated with receptor-targeting ligands; these cell-specific delivery strategies facilitate siRNA incorporation through endocytosis.

Table 1 | **Delivery methods for RNA-interference-based therapeutics**

Method	Nucleic acid delivered	Advantages	Disadvantages
Non-viral delivery			
Cholesterol	siRNA	Systemic delivery, stable	Non-selective delivery
SNALP	siRNA	Systemic delivery, highly stable	Non-selective delivery
Fab	siRNA	Receptor-specific delivery	Relatively complex formulation
Aptamer	siRNA	Receptor-specific delivery	Large-scale sequence screening required
Nanoparticle	siRNA	Receptor-specific, self-assembling	Sophisticated preparation required
Viral delivery			
Lentivirus	RNA (shRNA produced)	Stable expression, transduces non-dividing cells	Gene-disruption risk, localized delivery
Adenovirus	dsDNA (shRNA produced)	Episomal, no insertional mutagenesis	Immunogenic, dose-dependent hepatotoxicity
AAV	ssDNA/dsDNA (shRNA produced)	Episomal, low genomic integration	Immunogenic, small vector capacity

AAV, adeno-associated virus; dsDNA, double-stranded DNA; Fab, heavy-chain antibody fragment; shRNA, short hairpin RNA; siRNA, small interfering RNA; SNALP, stable nucleic acid–lipid particle; ssDNA, single-stranded DNA.

Heavy-chain antibody fragments (Fabs) that are specific for the HIV-1 envelope glycoprotein gp120 have been used to achieve selective delivery of siRNAs to HIV-infected cells both in culture and *in vivo*[73] (FIG. 5b). Fab molecules were conjugated to positively charged protamine, which interacts electrostatically with negatively charged siRNA molecules, and fluorescent tagging was used to monitor the specificity of targeting to infected cells. Fab–siRNA complexes targeting the HIV mRNA for the group-specific antigen protein (Gag) were successfully incorporated into cultured T lymphocytes infected with HIV-1, resulting in a >70% decrease in the levels of p24 Gag. The ERBB2 growth factor receptor was also targeted effectively with a Fab–siRNA conjugate in a breast cancer cell line, further illustrating the utility of this approach for cell-type-specific delivery of siRNAs.

Structured RNA ligands known as aptamers can be designed to bind with specific cell-surface receptors and can be linked covalently to siRNAs for cell-type-specific delivery *in vivo*; the siRNA molecules are released from the aptamer on entering the cell. In one study, aptamers that bind specifically to the prostate-specific membrane antigen (PSMA), which is expressed on the surface of prostate cancer cells, were conjugated to siRNAs and facilitated the reduction of tumour growth in a mouse model after intratumoral injections every 2 days for 20 days[74]. Another study using an aptamer-based approach also targeted cells expressing PSMA, using biotinylated siRNAs and biotinylated aptamers bound together non-covalently by the biotin-binding protein streptavidin[75] (FIG. 5b). Biotin-labelled 27mer siRNAs were incorporated into these aptamer complexes to
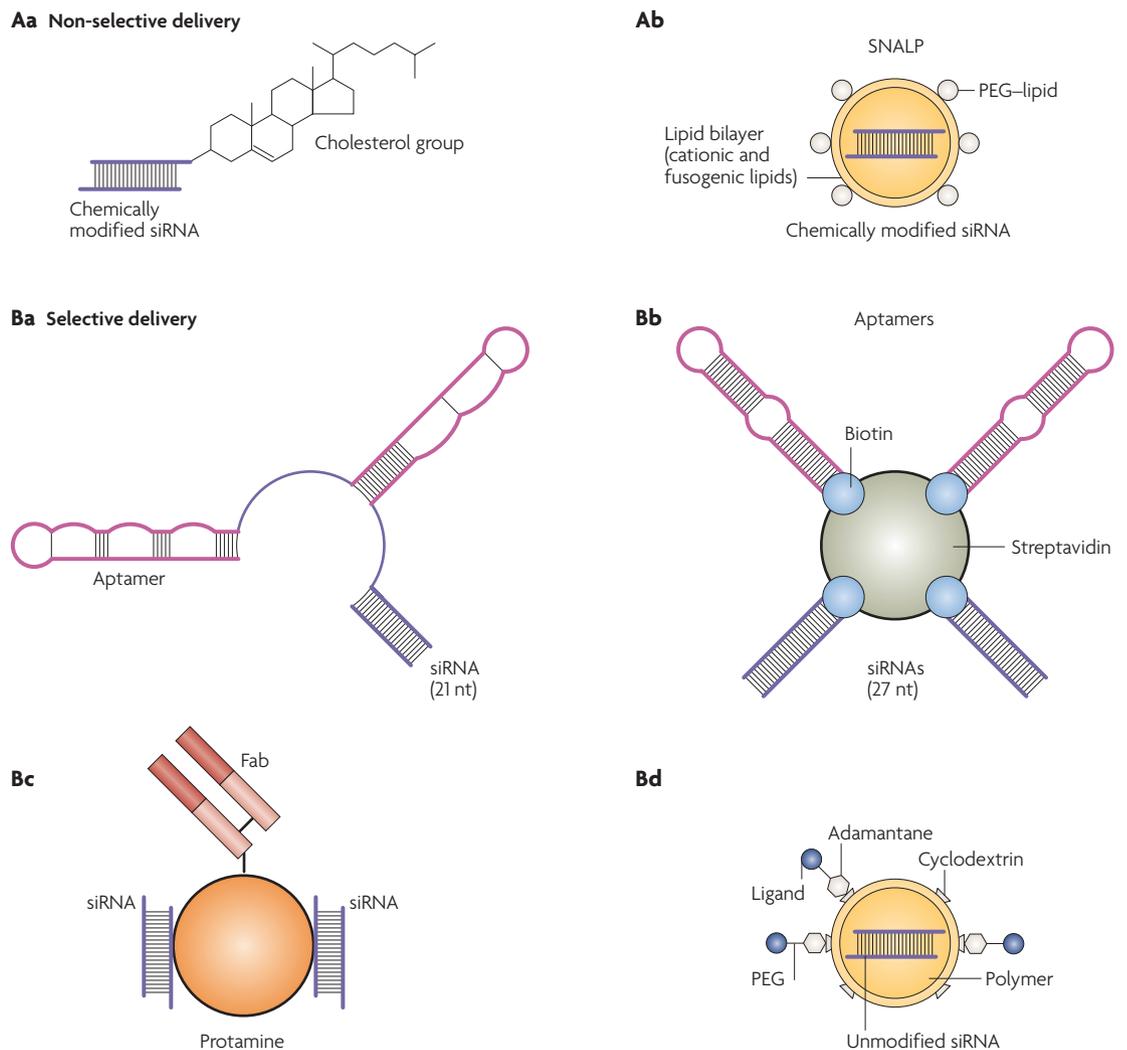

Figure 5 | **Delivery of small interfering RNAs. A** | Non-selective approaches. Cholesterol groups can be linked to chemically modified small interfering RNAs (siRNAs) for systemic delivery (**Aa**). siRNAs can also be delivered systemically by stable nucleic acid–lipid particles (SNALPs) (**Ab**). **B** | Selective approaches. Aptamer–siRNA chimaeras allow siRNAs to be delivered to specific cell types that display receptors recognized by the aptamers (**Ba** and **Bb**). An all-RNA approach can be used to couple aptamers and siRNAs (**Ba**), or a biotin–streptavidin approach can be used to achieve coupling (**Bb**). Heavy-chain antibody fragments (Fabs) and siRNAs can be linked with protamine to deliver siRNAs to specific cell-surface receptors (**Bc**). Nanoparticles that display specific ligands on their surfaces can be used to target siRNAs to particular cell types (**Bd**). PEG, polyethylene glycol.
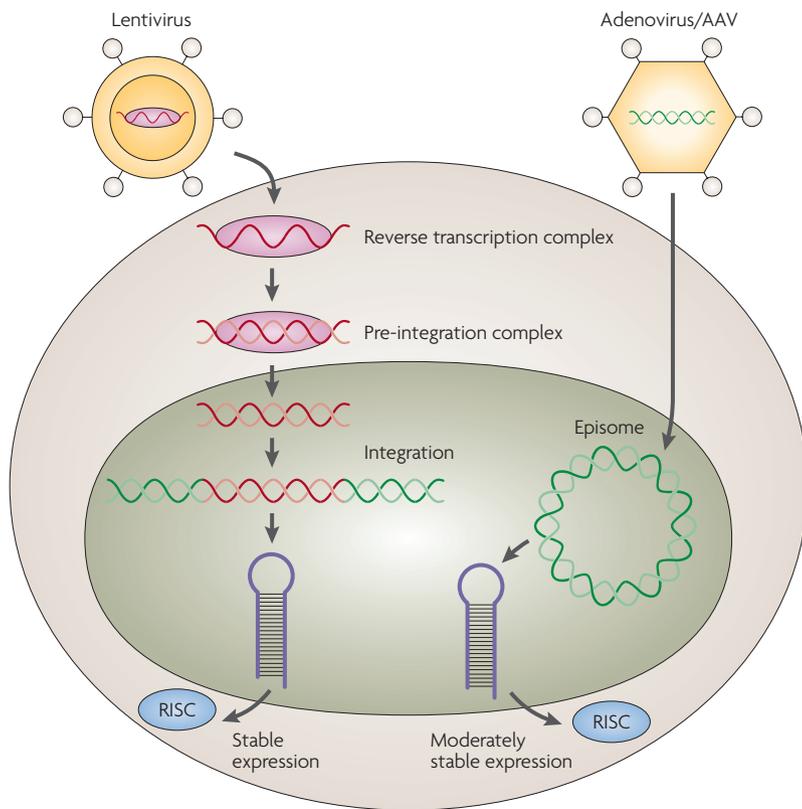
Figure 6 | **Viral delivery of short hairpin RNAs.** Lentiviral vectors are used to deliver therapeutic, short hairpin RNA (shRNA)-expressing transgenes that integrate into the genome for stable shRNA expression. Adenoviral and adeno-associated virus (AAV) vectors fail to integrate their transgenes into the genome but instead express shRNAs episomally for moderately stable levels of shRNA expression. RISC, RNA-induced silencing complex.

induce a more potent RNAi response by incorporating the Dicer step in RISC loading and activation.

Nanoparticles provide another powerful approach to selective systemic delivery of RNAi-inducing molecules. These vehicles are designed to carry large, protected payloads of siRNAs to target cells in a highly specific manner. Targeting is achieved by coating nanoparticle surfaces with cell-type-specific ligands. In an important proof-of-concept study, Ewing sarcoma tumours were targeted *in vivo* using nanoparticles coated with transferrin ligands[76]. These nanoparticles were composed of cyclodextrin-containing polycations (CDPs) engineered to incorporate negatively charged siRNAs. PEG polymers were coupled to the outer surface of these nanoparticles through terminal adamantane groups on the polymers for added stability and to prevent possible aggregation, which would limit their efficacy *in vivo*. Adamantane–PEG chains were covalently linked to transferrin ligands, and adamantane–PEG chains and linear CDPs were designed for self-assembly into uniformly sized nanoparticles (~50 nanometres in diameter) for selective systemic delivery (FIG. 5b). Several injections of the transferrin receptor-targeting nanoparticles carrying siRNAs directed against the *Ews–Fli1* (Ewing sarcoma breakpoint region 1–flightless 1 homologue) fusion gene resulted in the inhibition of tumour formation in a mouse xenograph model.

Episome
A double-stranded, circular DNA molecule that replicates in the nucleus without integrating into the host genome.

Pseudotyping
Changing the ability of a viral vector to bind cell-surface receptors by altering its envelope proteins.

Viral delivery of RNAi-inducing agents

Chronic diseases require long-term RNAi, which must be mediated by viral expression vectors (TABLE 1); this involves a gene therapy approach to incorporate shRNA transgenes into cells for genomic integration or episomal expression. Gene therapy vectors have been under development for the past two decades, and safety concerns that remain regarding their use also apply to RNAi-based therapies. Targeting viral vectors to specific cell types and minimizing their toxicities are vital concerns; however, recent studies illustrate the potential efficacy of viral vector-delivered RNAi.

Lentiviral vectors transduce both dividing and non-dividing cells, allowing stable shRNA expression from transgene integration into the host cell genome (FIG. 6). Commonly derived from HIV or feline immunodeficiency virus, lentiviral vectors are often pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) to increase the range of cell types that can be transduced. One application of RNAi to be used in an upcoming clinical trial involves *ex vivo* lentiviral vector delivery of an shRNA expression cassette into haematopoietic stem cells collected from patients infected with HIV; the transduced cells are to be reinfused into these patients for therapeutic benefit *in vivo*[77].

Vectors derived from adenoviruses or AAVs are used for more transient expression of shRNAs in therapeutic strategies against cancers and other diseases in which long-term RNAi is not desired. These non-integrating vectors largely remain episomal, integrating at low frequencies, and transduce both dividing and non-dividing cells with therapeutic shRNA transgenes[78] (FIG. 6). A possible drawback to both adenoviral-based and AAV-based vectors is that repeated administrations can trigger strong immune responses, thereby potentially limiting their effectiveness in certain therapeutic settings.

Specific therapeutic applications of RNAi

Viral diseases. The therapeutic potential of RNAi was first highlighted in experiments demonstrating the efficacy of this approach against HIV infection in cultured cells[79–82]. Now, just a few years later, clinical trials using RNAi-based strategies are under way for respiratory syncytial virus (RSV) infection and are soon to begin for HIV infection (TABLE 2). Viral RNAs and replication intermediates are potential targets for RNAi-based therapies, and most viral transcripts in cells infected with HIV have already been effectively targeted using RNAi[7]. Cellular genes required for HIV entry or replication have also been targeted as an alternative strategy, because this avoids the problem of genetic variability in HIV — as few as one or two mismatches in siRNA cleavage sites dramatically decrease the potency of RNAi[83]. The HIV co-receptor CC chemokine receptor 5 (CCR5) is a particularly promising target for RNAi-based therapy, as individuals who are homozygous for a 32-bp deletion in this gene are resistant to HIV infection and do not suffer adverse effects in immune function[84]. Highly conserved sequences in the viral genome are also promising candidates for therapeutic RNAi. A combinatorial approach using a single shRNA, an RNA decoy of the HIV TAR element found on

viral transcripts, and a hammerhead ribozyme has recently been shown to have long-term efficacy against HIV infection in primary cells[85]. This triple RNA therapy will be delivered to haematopoietic stem cells *ex vivo* using lentiviral vectors in an impending HIV clinical trial.

For HBV infection, delivery of siRNAs *in vivo* using SNALPs has been used to reduce viral replication in mice[70]. In this study, extensive siRNA chemical modifications prevented interferon responses and stabilized the siRNAs in serum, and the therapeutic effect of one dose of siRNAs persisted for up to 1 week. In another study, low doses of AAV expression vectors carrying anti-HBV shRNAs persistently inhibited HBV over a period of 5 months in mice[13], demonstrating stable and effective RNAi against viral infections.

Intranasal delivery of siRNAs has been shown to be highly effective as a method for inhibiting RSV infection in mice, and the promise of this mode of delivery is evidenced by an ongoing RSV clinical trial in humans. In one initial study, siRNAs delivered intranasally using either nanochitosan polymer-based nanoparticles or a transfection reagent, or administered alone, effectively elicited RNAi without inducing an interferon response[86]. A second intranasal siRNA delivery study targeting both parainfluenza virus and RSV concurrently[6] demonstrated competition between siRNAs effective against two different viral targets. Whether competition resulted from saturation of RISC effector complexes or another upstream component of the RNAi pathway is not clear, but such siRNA overloading should be avoided in therapeutic strategies against multiple targets.

Studies of vaginal transmission of herpes simplex virus 2 (HSV-2) in mice showed that infection can be blocked using a siRNA microbicide[87]. Vaginal applications of lipid-encapsulated, unmodified siRNAs targeting HSV-2 genes were tested in mice, and the therapeutic effect of siRNAs administered before or after exposure

to virus was assessed during a 15-day period. Six days after infection, 70% of mice treated with one type of siRNA before viral exposure showed no signs of HSV-2 infection. However, after viral challenge, a combination of two siRNAs with distinct viral targets was required to protect against HSV-2 infection. These results indicate that lipid-encapsulated siRNAs can be used as an effective microbicide at mucosal surfaces, with no apparent toxicities *in vivo*. Taken together with the intranasal delivery studies, mucosal membranes seem to be effective sites for siRNA delivery; this approach should prove to be a useful platform for therapeutic delivery of siRNAs in terms of both accessibility and cost-effectiveness.

Ocular diseases. In two ongoing RNAi clinical trials, direct intravitreal injections of siRNAs targeting vascular endothelial growth factor (VEGF) or its receptor (VEGFR1) have been performed to test for their safety and efficacy in the eye. Ocularly delivered siRNAs targeting VEGF (Acuity Pharmaceuticals) and VEGFR1 (Sirna Therapeutics) are currently in the early stages of clinical trials for the treatment of AMD and so far no adverse events have been reported in patients. This direct injection approach might also prove useful for other ocular diseases.

Neurodegenerative diseases. Delivery of RNAi into the mouse brain has been used effectively to treat spinocerebellar ataxia type 1 (SCA1), a dominantly inherited disease that is part of a group of neurodegenerative disorders that includes Huntington disease. SCA1 is caused by expanded CAG trinucleotide repeats in the mutant form of the SCA1 gene (*Sca1*; also known as *Atxn1*), which generates polyglutamine (polyQ) expansions. The accumulation of defective polyQ gene products is toxic to neuronal cells, making this an ideal target for RNAi-mediated knockdown. Knockdown of polyQ products in mice was achieved using AAV shRNA vectors injected

Hammerhead ribozyme
Small, self-cleaving catalytic RNAs with distinct secondary structures and highly conserved core residues that mediate cleavage.

Table 2 | **Development of RNA-interference-based therapies**

Disease	Stage	RNAi reagent	Delivery	Company/institution
Ocular diseases				
AMD	Preclinical stage	siRNA	Direct intravitreal injection	Quark Biotech
	Clinical trial phase I	siRNA	Direct intravitreal injection	Sirna
	Clinical trial phase II	siRNA	Direct intravitreal injection	Acuity
Viral infections				
Hepatitis B and C	Preclinical stage	shRNA	Liganded nanoparticle	Nucleonics/Intradigm
RSV	Clinical trial phase I	siRNA	Aerosol	Alnylam
HIV	Clinical trial phase I (scheduled for 2007)	shRNA	Lentivirus	Benitec/City of Hope
Cancer				
Hepatic cancer	Preclinical stage	siRNA	Liganded nanoparticle	Calando
Solid tumour cancers	Preclinical stage	siRNA	Liganded nanoparticle	Intradigm
Other disease types				
ALS	Preclinical stage	siRNA	N/A	CytRx
Inflammatory diseases	Preclinical stage	siRNA	Peptide	Nastech

ALS, amyotrophic lateral sclerosis; AMD, age-related macular degeneration; RNAi, RNA interference; RSV, respiratory syncytial virus; shRNA, short hairpin RNA; siRNA, small interfering RNA.

into the brains of SCA1 mice, and led to improvements in the pathology of neuronal cells, even under conditions that showed low transduction efficiencies[88].

Other studies have focused on mouse models of another neurodegenerative disease, amyotrophic lateral sclerosis (ALS). Lentiviral vector delivery of RNAi against the mutant superoxide dismutase 1 (*Sod1*) gene led to long-term, stable gene silencing, along with improved survival of motor neurons and delayed onset of the disease phenotype in mice[89,90]. RNAi-based therapeutic strategies against SCA1 and ALS show the effectiveness of viral delivery methods *in vivo* without toxic, vector-related side effects.

Cancer. Oncogenes expressed at abnormally high levels are attractive targets for RNAi-based therapies against cancers[91], and such approaches have effectively inhibited tumour growth *in vivo* in mouse models. One successful study involved liposomal delivery of siRNAs targeting the tyrosine kinase receptor *EphA2* gene, which is overexpressed in ovarian cancer cells[92]. After biweekly delivery of siRNAs for 4 weeks, an up to 50% reduction of tumour size was observed. When RNAi therapy was combined with the chemotherapy agent paclitaxel, an up to 90% reduction in tumour size was observed, indicating the potency and effectiveness of combining RNAi with conventional forms of therapy, especially for cancers.

As mentioned above, metastatic Ewing sarcoma cells have been successfully targeted in a mouse model using cyclodextrin nanoparticles to systemically deliver siRNAs targeting the *Ews–Fli1* gene fusion[76]. Tumour growth *in vivo* was suppressed after systemic delivery of siRNA-containing nanoparticles. Of greater significance, however, was that the high rate of relapse associated with traditional chemotherapy treatments for these tumour cells was not observed in mice injected with siRNA nanoparticles, indicating the potential long-term therapeutic benefit of this highly selective, systemic RNAi approach in the treatment of cancers.

Conclusions and future perspectives

The significance of the discovery of RNAi is highlighted by the fact that the 2006 Nobel Prize in Physiology or Medicine was awarded to its discoverers, Andrew Fire and Craig Mello. Not only has RNAi had a profound effect on studies of gene regulation, it has also led to the development of a new class of therapeutic agents based on small dsRNAs[93]. RNAi-based therapies for AMD and RSV have already reached clinical trials, and, within the next year or two, trials will also have begun for HIV, HBV, hepatitis C virus and Huntington disease. The rapid progression from basic discovery to applications in medicine is unprecedented, and is indicative of the enormous therapeutic potential of RNAi.

As with all new technologies that progress at a rapid pace, roadblocks to therapeutic applications have been encountered. Some of the early setbacks for RNAi included demonstrations of interferon induction and OTEs mediated by siRNAs. More recent findings have shown that high levels of promoter-based shRNA expression can be toxic and fatal in mice. However, as discussed

in this Review, solutions to these problems have quickly been developed in the form of simple backbone modifications to siRNAs and appropriate promoter choices for shRNAs. Although systemic delivery was at first thought to be the main obstacle preventing rapid development of siRNA-based drugs, the arsenal of delivery methods that were developed for antisense oligos and plasmid DNAs were tested and shown to have utility for RNAi-based therapies. Recent developments in delivery have also facilitated cell-specific targeting of RNAi reagents through the use of ligands such as receptor-targeting aptamers or ligand-coated nanoparticles.

However, there are still a number of major concerns and possible impediments to the widespread application of RNAi for the treatment of human disease. For instance, chronic diseases such as hepatitis C virus or HIV infections will require lifelong treatments with RNAi. Not enough is known about the potential downside of prolonged or repetitive use of RNAi triggers on normal cellular metabolism. In terms of long-term applications of siRNAs, it is entirely feasible that toxicities will not show up for months, or perhaps even years. There is also the emerging area of TGS mediated by siRNAs. Because siRNAs/shRNAs that direct PTGS are complementary to both the message and the gene, it is possible that unwanted chromatin changes could be directed by long-term use of siRNAs/shRNAs that mediate PTGS. Investigating such issues would require long-term studies in therapeutically relevant animal models of RNAi.

Cancer is clearly an important potential target for RNAi-based therapies, but the main challenge in cancer therapy is targeting metastatic cells that have spread from the original tumour. With other forms of chemotherapy, these cells often become refractory to treatment, resulting in relapse. The jury is still out on whether siRNAs can be effectively delivered to such cells and whether appropriate targets can be identified to destroy the metastatic population. In addition, neurodegenerative diseases such as Huntington or ALS are also attractive targets for RNAi, because there are no effective therapies for these debilitating conditions. For such diseases, the primary challenge is delivery of RNAi to specific cells in the nervous system; siRNA delivery vehicles have not yet been shown to cross the blood–brain barrier. Direct injection into the brain has been the approach used in experimental animal models so far, but there is likely to be resistance to the use of this approach in humans owing to the invasive nature of brain delivery and the possible need for continual applications of siRNAs, although viral delivery of shRNAs would avoid the requirement for multiple siRNA doses.

There are also other potential reservations and problems that might delay or even prevent RNAi-based therapies for the treatment of certain conditions; however, this remains the single most promising approach for a broad spectrum of diseases. Clearly, further studies must be carried out to achieve effective delivery and to better understand the unwanted side effects of RNAi-based therapies. Given the immense interest in RNAi as a potential therapeutic, the coming years are likely to see the detailed investigation of these issues and an increasing range of applications for RNAi-based treatments.

1. Fire, A. *et al.* Potent and specific genetic interference by double stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811 (1998).
   This Nobel Prize-winning landmark paper provides the first description of the phenomenon of RNAi.

2. Elbashir, S. M. *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498 (2001).
   This work provides the first description of RNAi in mammalian cells.

3. Hannon, G. J. & Rossi, J. J. Unlocking the potential of the human genome with RNA interference. *Nature* **431**, 371–378 (2004).

4. Check, E. A crucial test. *Nature Med.* **11**, 243–244 (2005).

5. McFarland, T. J., Zhang, Y., Appukuttan, B. & Stout, J. T. Gene therapy for proliferative ocular diseases. *Expert Opin. Biol. Ther.* **4**, 1053–1058 (2004).

6. Bitko, V., Musiyenko, A., Shulyayeva, O. & Barik, S. Inhibition of respiratory viruses by nasally administered siRNA. *Nature Med.* **11**, 50–55 (2005).

7. Rossi, J. J. RNAi as a treatment for HIV-1 infection. *Biotechniques* **40**, s25–s29 (2006).

8. Dykxhoorn, D. M. & Lieberman, J. Silencing viral infection. *PLoS Med.* **3**, e242 (2006).

9. Raoul, C., Barker, S. D. & Aebischer, P. Viral-based modeling and correction of neurodegenerative diseases by RNA interference. *Gene Ther.* **13**, 487–495 (2006).

10. Pai, S. I. *et al.* Prospects of RNA interference therapy for cancer. *Gene Ther.* **13**, 464–477 (2006).

11. Amarzguioui, M., Rossi, J. J. & Kim, D. Approaches for chemically synthesized siRNA and vector-mediated RNAi. *FEBS Lett.* **579**, 5974–5981 (2005).

12. de Veer, M. J., Sledz, C. A. & Williams, B. R. Detection of foreign RNA: implications for RNAi. *Immunol. Cell Biol.* **83**, 224–228 (2005).

13. Grimm, D. *et al.* Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**, 537–541 (2006).
    This paper describes the *in vivo* toxicity of long-term, high-level shRNA expression in mice.

14. Behlke, M. A. Progress towards *in vivo* use of siRNAs. *Mol. Ther.* **13**, 644–670 (2006).

15. Meister, G. & Tuschl, T. Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**, 343–349 (2004).

16. Matzke, M. A. & Birchler, J. A. RNAi-mediated pathways in the nucleus. *Nature Rev. Genet.* **6**, 24–35 (2005).

17. Wassenegger, M. The role of the RNAi machinery in heterochromatin formation. *Cell* **122**, 13–16 (2005).

18. Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33 (2000).

19. Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366 (2001).

20. Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R. & Tuschl, T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110**, 563–574 (2002).

21. Liu, J. *et al.* Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**, 1437–1441 (2004).

22. Matranga, C., Tomari, Y., Shin, C., Bartel, D. P. & Zamore, P. D. Passenger-strand cleavage facilitates assembly of siRNA into AGO2-containing enzyme complexes. *Cell* **123**, 607–620 (2005).

23. Rand, T. A., Petersen, S., Du, F. & Wang, X. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* **123**, 621–629 (2005).

24. Parker, J. S., Roe, S. M. & Barford, D. Structural insights into mRNA recognition from a PIWI domain–siRNA guide complex. *Nature* **434**, 663–666 (2005).

25. Ma, J. B., Ye, K. & Patel, D. J. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature* **429**, 318–322 (2004).

26. Elbashir, S. M., Lendeckel, W. & Tuschl, T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* **15**, 188–200 (2001).

27. Orban, T. I. & Izaurralde, E. Decay of mRNAs targeted by RISC requires XRN1 and the Ski complex, and the exosome. *RNA* **11**, 459–469 (2005).

28. Hutvagner, G. & Zamore, P. D. A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297**, 2056–2060 (2002).

29. Bartel, D. P. & Chen, C. Z. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nature Rev. Genet.* **5**, 396–400 (2004).

30. Lee, R. C., Feinbaum, R. L. & Ambros, V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* **75**, 843–854 (1993).

31. Wightman, B., Ha, I. & Ruvkun, G. Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell* **75**, 855–862 (1993).

32. Liu, J., Valencia-Sanchez, M. A., Hannon, G. J. & Parker, R. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nature Cell Biol.* **7**, 719–723 (2005).

33. Yekta, S., Shih, I. H. & Bartel, D. P. MicroRNA-directed cleavage of *Hoxb8* mRNA. *Science* **304**, 594–596 (2004).

34. Lee, Y. *et al.* MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* **23**, 4051–4060 (2004).

35. Borchert, G. M., Lanier, W. & Davidson, B. L. RNA polymerase III transcribes human microRNAs. *Nature Struct. Mol. Biol.* **13**, 1097–1101 (2006).

36. Lee, Y. *et al.* The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415–419 (2003).

37. Gregory, R. I. *et al.* The Microprocessor complex mediates the genesis of microRNAs. *Nature* **432**, 235–240 (2004).

38. Yi, R., Qin, Y., Macara, I. G. & Cullen, B. R. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev.* **17**, 3011–3016 (2003).

39. Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E. & Kutay, U. Nuclear export of microRNA precursors. *Science* **303**, 95–98 (2004).

40. Chendrimada, T. P. *et al.* TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436**, 740–744 (2005).

41. Lee, Y. *et al.* The role of PACT in the RNA silencing pathway. *EMBO J.* **25**, 522–532 (2006).

42. Preall, J. B. & Sontheimer, E. J. RNAi: RISC gets loaded. *Cell* **123**, 543–245 (2005).

43. Lewis, B. P., Burge, C. B. & Bartel, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**, 15–20 (2005).

44. Silva, J. M. *et al.* Second-generation shRNA libraries covering the mouse and human genomes. *Nature Genet.* **37**, 1281–1288 (2005).

45. Morris, K. V., Chan, S. W., Jacobsen, S. E. & Looney, D. J. Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **305**, 1289–1292 (2004).

46. Kim, D. H., Villeneuve, L. M., Morris, K. V. & Rossi, J. J. Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. *Nature Struct. Mol. Biol.* **13**, 793–797 (2006).

47. Janowski, B. A. *et al.* Involvement of AGO1 and AGO2 in mammalian transcriptional silencing. *Nature Struct. Mol. Biol.* **13**, 787–792 (2006).

48. Ting, A. H., Schuebel, K. E., Herman, J. G. & Baylin, S. B. Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. *Nature Genet.* **37**, 906–910 (2005).

49. Weinberg, M. S. *et al.* The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. *RNA* **12**, 256–262 (2006).

50. Vastenhouw, N. L. *et al.* Gene expression: long-term gene silencing by RNAi. *Nature* **442**, 882 (2006).

51. Kim, D. H. *et al.* Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nature Biotechnol.* **23**, 222–226 (2005).

52. Siolas, D. *et al.* Synthetic shRNAs as potent RNAi triggers. *Nature Biotechnol.* **23**, 227–231 (2005).

53. Marques, J. T. *et al.* A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nature Biotechnol.* **24**, 559–565 (2006).

54. Brummelkamp, T. R., Bernards, R. & Agami, R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550–553 (2002).

55. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J. & Conklin, D. S. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* **16**, 948–958 (2002).
    References 54 and 55 describe stable expression systems for RNAi.

56. Zeng, Y., Wagner, E. J. & Cullen, B. R. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol. Cell* **9**, 1327–1333 (2002).

57. Sarnow, P., Jopling, C. L., Norman, K. L., Schutz, S. & Wehner, K. A. MicroRNAs: expression, avoidance and subversion by vertebrate viruses. *Nature Rev. Microbiol.* **4**, 651–659 (2006).

58. Jackson, A. L. *et al.* Widespread siRNA 'off-target' transcript silencing mediated by seed region sequence complementarity. *RNA* **12**, 1179–1187 (2006).

59. Birmingham, A. *et al.* 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nature Methods* **3**, 199–204 (2006).

60. Hornung, V. *et al.* Sequence-specific potent induction of IFN-α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nature Med.* **11**, 263–270 (2005).
    This work describes the detection of immunostimulatory motifs in siRNA sequences through TLRs.

61. Manche, L., Green, S. R., Schmedt, C. & Mathews, M. B. Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol. Cell. Biol.* **12**, 5238–5248 (1992).

62. Seth, R. B., Sun, L. & Chen, Z. J. Antiviral innate immunity pathways. *Cell Res.* **16**, 141–147 (2006).

63. Judge, A. D. *et al.* Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nature Biotechnol.* **23**, 457–462 (2005).

64. Kim, D. H. *et al.* Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. *Nature Biotechnol.* **22**, 321–325 (2004).

65. Robbins, M. A. *et al.* Stable expression of shRNAs in human CD34+ progenitor cells can avoid induction of interferon responses to siRNAs *in vitro*. *Nature Biotechnol.* **24**, 566–571 (2006).

66. An, D. S. *et al.* Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol. Ther.* **14**, 494–504 (2006).

67. Czauderna, F. *et al.* Structural variations and stabilizing modifications of synthetic siRNAs in mammalian cells. *Nucleic Acids Res.* **31**, 2705–2716 (2003).

68. Morrissey, D. V. *et al.* Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. *Hepatology* **41**, 1349–1356 (2005).

69. Chiu, Y. L. & Rana, T. M. siRNA function in RNAi: a chemical modification analysis. *RNA* **9**, 1034–1048 (2003).

70. Morrissey, D. V. *et al.* Potent and persistent *in vivo* anti-HBV activity of chemically modified siRNAs. *Nature Biotechnol.* **23**, 1002–1007 (2005).

71. Soutschek, J. *et al.* Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**, 173–178 (2004).
    This work describes a straightforward and effective method for intravenous systemic delivery of RNAi.

72. Zimmermann, T. S. *et al.* RNAi-mediated gene silencing in non-human primates. *Nature* **441**, 111–114 (2006).
    This paper describes the potency and longevity of systemic delivery of RNAi in non-human primates.

73. Song, E. *et al.* Antibody mediated *in vivo* delivery of small interfering RNAs via cell-surface receptors. *Nature Biotechnol.* **23**, 709–717 (2005).

74. McNamara, J. O. *et al.* Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nature Biotechnol.* **24**, 1005–1015 (2006).

75. Chu, T. C., Twu, K. Y., Ellington, A. D. & Levy, M. Aptamer mediated siRNA delivery. *Nucleic Acids Res.* **34**, e73 (2006).

76. Hu-Lieskovan, S., Heidel, J. D., Bartlett, D. W., Davis, M. E. & Triche, T. J. Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer Res.* **65**, 8984–8992 (2005).

77. Morris, K. V. & Rossi, J. J. Lentiviral-mediated delivery of siRNAs for antiviral therapy. *Gene Ther.* **13**, 553–558 (2006).

78. Grimm, D. & Kay, M. A. Therapeutic short hairpin RNA expression in the liver: viral targets and vectors. *Gene Ther.* **13**, 563–575 (2006).

79. Lee, N. S. *et al.* Expression of small interfering RNAs targeted against HIV-1 *rev* transcripts in human cells. *Nature Biotechnol.* **20**, 500–505 (2002).

80. Novina, C. D. *et al.* siRNA-directed inhibition of HIV-1 infection. *Nature Med.* **8**, 681–686 (2002).

81. Jacque, J., Triques, K. & Stevenson, M. Modulation of HIV-1 replication by RNA interference. *Nature* **418**, 435–438 (2002).

82. Coburn, G. A. & Cullen, B. R. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. *J. Virol.* **76**, 9225–9231 (2002).

83. Martinez, J. & Tuschl, T. RISC is a 5′ phosphomonoester-producing RNA endonuclease. *Genes Dev.* **18**, 975–980 (2004).

84. Huang, Y. *et al.* The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nature Med.* **2**, 1240–1243 (1996).

85. Li, M. J. *et al.* Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. *Mol. Ther.* **12**, 900–909 (2005).

86. Zhang, W. *et al.* Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. *Nature Med.* **11**, 56–62 (2005).

87. Palliser, D. *et al.* An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. *Nature* **439**, 89–94 (2006).
This paper describes the efficacy of topical delivery of RNAi as a microbicide.

88. Xia, H. *et al.* RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. *Nature Med.* **10**, 816–820 (2004).

89. Raoul, C. *et al.* Lentiviral-mediated silencing of *Sod1* through RNA interference retards disease onset and progression in a mouse model of ALS. *Nature Med.* **11**, 423–428 (2005).

90. Ralph, G. S. *et al.* Silencing mutant *Sod1* using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nature Med.* **11**, 429–433 (2005).

91. Pai, S. I. *et al.* Prospects of RNA interference therapy for cancer. *Gene Ther.* **13**, 464–477 (2006).

92. Landen, C. N. *et al.* Therapeutic *EphA2* gene targeting *in vivo* using neutral liposomal small interfering RNA delivery. *Cancer Res.* **65**, 6910–6918 (2005).

93. Dykxhoorn, D. M. & Lieberman, J. Knocking down disease with siRNAs. *Cell* **126**, 231–235 (2006).

DATABASES
The following terms in this article are linked online to:
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
AGO2 | APOB | ATF2 | CCR5 | DGCR8 | Dicer | exportin 5 | *Hoxb8* | PKR | *Sod1* | TLR3/7/8 | VEGF
OMIM: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
ALS | SCA1

FURTHER INFORMATION
Ambion RNAi Interference Resource:
http://www.ambion.com/techlib/resources/RNAi/
Gene Link RNAi Explorer:
http://www.genelink.com/sirna/shRNAi.asp
Integrated DNA Technologies RNAi design tools: http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx/
Invitrogen BLOCK-iT RNAi Designer:
https://rnaidesigner.invitrogen.com/rnaiexpress/
Promega siRNA Target Designer:
http://www.promega.com/siRNADesigner/program/
siRNA at the Whitehead Institute:
http://jura.wi.mit.edu/bioc/siRNAext/
Access to this links box is available online.