

# Interfering with disease: a progress report on siRNA-based therapeutics

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Abstract | RNA interference (RNAi) quietly crept into biological research in the 1990s when unexpected gene-silencing phenomena in plants and flatworms first perplexed scientists. Following the demonstration of RNAi in mammalian cells in 2001, it was quickly realized that this highly specific mechanism of sequence-specific gene silencing might be harnessed to develop a new class of drugs that interfere with disease-causing or disease-promoting genes. Here we discuss the considerations that go into developing RNAi-based therapeutics starting from *in vitro* lead design and identification, to *in vivo* pre-clinical drug delivery and testing. We conclude by reviewing the latest clinical experience with RNAi therapeutics.

RNAi therapeutics represent a fundamentally new way to treat human disease by addressing targets that are otherwise 'undruggable' with existing medicines. The discovery of long double-stranded RNA (dsRNA) mediated RNAi in the worm<sup>1</sup> and the subsequent demonstration that RNAi, mediated by small-interfering RNA (siRNA), operates in mammalian cells<sup>2</sup> sparked an explosion of research to uncover new mechanisms of gene silencing, revolutionizing our understanding of endogenous mechanisms of gene regulation and providing powerful new tools for biological research and drug discovery. From the first *in vivo* evidence of RNAi-based therapeutic efficacy in an animal disease model in 2003 (REF. 3), the pace of drug development has been rapid. Three phase I studies to investigate RNAi-based drugs for age-related macular degeneration (AMD), a leading cause of blindness, and for respiratory syncytial virus (RSV), the leading cause of paediatric hospitalizations in the United States today, have already been completed without untoward toxicity. Despite the promise, real hurdles need to be overcome to translate the therapeutic potential of RNAi into clinical reality. An important milestone will be the clear demonstration of efficacy in well-controlled human clinical studies.

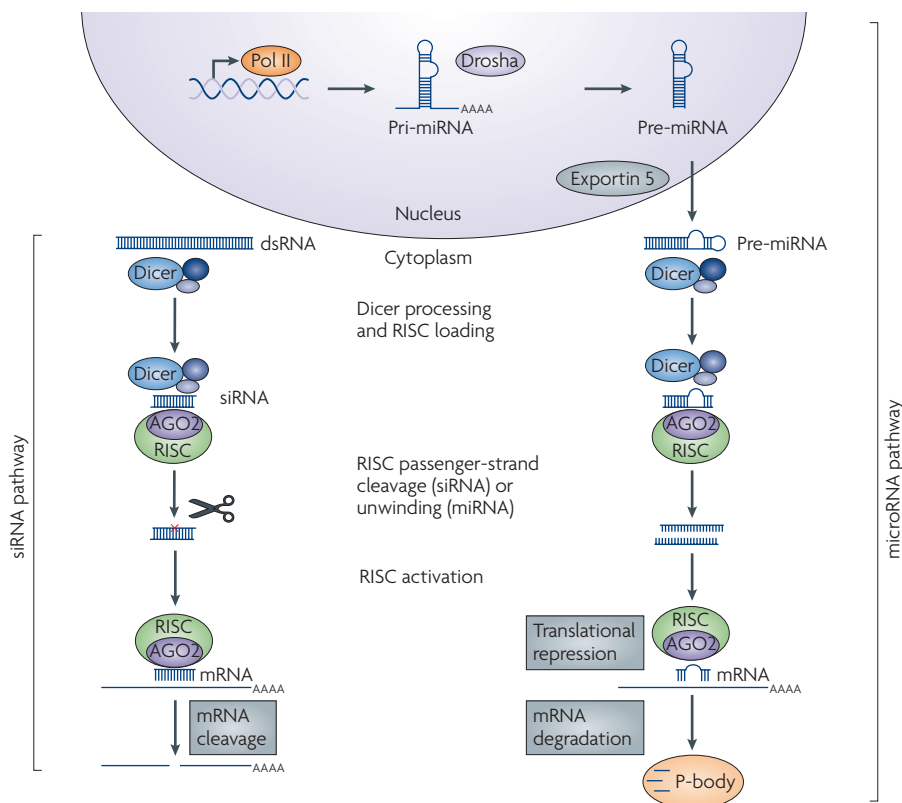
RNAi pathways are guided by small RNAs that include siRNAs and microRNAs (miRNAs), with the latter deriving from imperfectly paired non-coding hairpin RNA structures that are naturally transcribed by the genome<sup>4,5</sup>. Gene silencing can be induced by siRNA through sequence-specific cleavage of perfectly complementary messenger RNA (mRNA), whereas microRNAs mediate translational repression and transcript degradation for imperfectly complementary targets (FIG. 1). In the endogenous pathway, RNAs containing stem loops or short hairpin structures, encoded in intragenic regions or within introns, are processed in the nucleus and exported to the cytoplasm as precursor molecules called pre-microRNAs<sup>5,6</sup>. In the cytoplasm, the pre-microRNA is further shortened and processed by an RNase III enzyme called Dicer to produce an imperfectly matched, double-stranded microRNA. Dicer similarly processes long, perfectly matched dsRNA into siRNA (FIG. 1). A multi-enzyme complex, which includes Argonaute 2 (AGO2, also known as EIF2C2) and the RNA-induced silencing complex (RISC), binds to either the microRNA duplex or the siRNA duplex and discards one strand (it is thought the passenger or sense strand is cleaved in the case of siRNA and is released in the case of miRNA) to form an activated

complex containing the guide or antisense strand<sup>7</sup>. The activated AGO2-RISC complex then seeks and binds to an mRNA strand bearing a complementary sequence and inactivates its expression. If complementarity is less than perfect (such as with miRNA), the AGO2-RISC complex blocks translation. If complementarity is perfect or nearly perfect (such as with siRNA) the complex cleaves the mRNA strand between the nucleotides that are complementary to nucleotides 10 and 11 of the guide strand relative to the 5'-end (FIG. 1). In addition to translational repression, miRNAs may also mediate mRNA degradation in cytoplasmic compartments known as processing bodies (P-bodies)<sup>8</sup>. Gene silencing by mRNA cleavage is thought to be particularly potent, in part because mRNA cleavage leads to rapid nucleolytic degradation of the RNA fragments and frees the activated RISC complex to seek and destroy another target mRNA in a catalytic fashion<sup>9</sup>.

The goal of RNAi-based therapy is to activate selective mRNA cleavage for efficient gene silencing. It is possible to harness the endogenous pathway in one of two ways: either by using a viral vector to express short hairpin RNA (shRNA) that resembles microRNA precursors, or by introducing siRNAs that mimic the Dicer cleavage product into the cytoplasm. These siRNAs can bypass the earlier steps in the RNAi silencing pathway and can be loaded directly onto the AGO2-RISC complex. Although delivery of synthetic siRNA and viral delivery of shRNA are being developed as potential RNAi-based therapeutic approaches, this article focuses solely on the development of synthetic siRNA as drugs. Synthetic siRNAs harness the naturally occurring RNAi pathway in a manner that is consistent and predictable, thus making them particularly attractive as therapeutics. Moreover, as they enter the RNAi pathway later, siRNAs are less likely to interfere with gene regulation by endogenous microRNAs<sup>10</sup>. As a consequence, siRNAs are the class of RNAi therapeutics that are most advanced in preclinical and clinical studies.

## ***In vitro* selection**

The initial steps to identify potent lead siRNA candidates start with bioinformatics design and involve *in vitro* studies to determine



**Figure 1 | Mechanism of RNA interference in mammalian cells.** RNA interference (RNAi) pathways are guided by small RNAs that include small interfering RNA (siRNA) and microRNAs (miRNAs). The siRNA pathway begins with cleavage of long double-stranded RNA (dsRNA) by the Dicer enzyme complex into siRNA. These siRNAs are incorporated into Argonaute 2 (AGO2) and the RNAi-induced silencing complex (RISC). If the RNA duplex loaded onto RISC has perfect sequence complementarity, AGO2 cleaves the passenger (sense) strand so that active RISC containing the guide (antisense) strand is produced. The siRNA guide strand recognizes target sites to direct mRNA cleavage (carried out by the catalytic domain of AGO2). RNAi therapeutics developed to harness the siRNA pathway typically involve the delivery of synthetic siRNA into the cell cytoplasm. The microRNA pathway begins with endogenously encoded primary microRNA transcripts (pri-miRNAs) that are transcribed by RNA polymerase II (Pol II) and are processed by the Drosha enzyme complex to yield precursor miRNAs (pre-miRNAs). These precursors are then exported to the cytoplasm by exportin 5 and subsequently bind to the Dicer enzyme complex, which processes the pre-miRNA for loading onto the AGO2–RISC complex. When the RNA duplex loaded onto RISC has imperfect sequence complementarity, the passenger (sense) strand is unwound leaving a mature miRNA bound to active RISC. The mature miRNA recognizes target sites (typically in the 3'-UTR) in the mRNA, leading to direct translational inhibition. Binding of miRNA to target mRNA may also lead to mRNA target degradation in processing (P)-bodies. Modified with permission from *Nature Rev. Genet.* REF 5 © (2007) Macmillan Publishers Ltd.

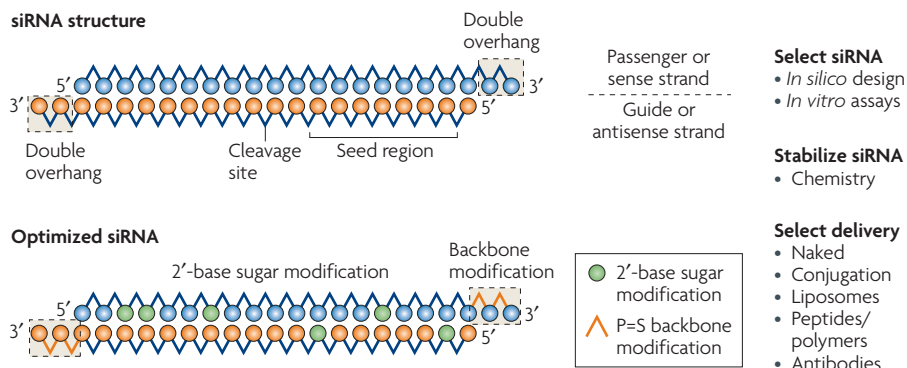
silencing efficacy, verify that unwanted off-target effects are absent and introduce chemical modifications, if needed, to improve stability and specificity. The overall scheme for turning siRNAs into drugs is summarized in FIG. 2. The three most important attributes to take into account when designing and selecting siRNA are potency, specificity and nuclease stability. Two types of off-target effects need to be avoided or minimized: silencing of genes sharing partial homology to the siRNA, and immune stimulation stemming from the recognition of certain siRNAs by the innate immune system. As our understanding of the molecular and

structural mechanism of RNAi has grown, the issues around lead siRNA selection are better understood and the off-target effects can generally be mitigated. It is now possible to rapidly identify (in the span of several weeks to months) potent, specific and stable *in vitro* active lead siRNA candidates targeting any gene of interest.

**Potency.** RNAi can be activated in mammalian cells by introducing siRNAs that mimic Dicer-cleaved, endogenous microRNAs. siRNAs can be identified to silence any gene, often with *in vitro* activity at nanomolar or lower concentrations. Algorithms to predict

effective siRNAs, based on common features of siRNAs identified by empirical testing, are available on the web<sup>11,12</sup>. Although algorithms can increase the chances of identifying active siRNA, they are imperfect and can sometimes miss the most potent siRNAs, which can only be identified by experimental testing. An alternative to using algorithms as a starting point to identify candidate siRNAs, is to test all the tiled 21 nucleotide sequences from the mRNA coding region experimentally and select a group of candidates that induce effective silencing at the lowest concentrations. One study suggests that slightly longer siRNAs, which need to be processed by Dicer before they can be incorporated into RISC, can be used as Dicer substrates and maintain silencing activity, although these longer siRNAs are more complicated to synthesize and could have an increased propensity to activate untoward immune responses<sup>13,14</sup>. Loading of RISC with respect to the sense and antisense siRNA strands is not symmetrical<sup>15–17</sup>. The strand that is least tightly bound at its 5'-end is favoured to bind to a deep pocket in RISC and become the active strand. Strand selection can in fact be manipulated by making a single nucleotide substitution at the end of the duplex to alter the relative binding of the ends<sup>15</sup>. By designing an siRNA with a mismatch at the 5'-end of the intended active strand that favours its incorporation into RISC, it may be possible to increase the likelihood of identifying a potent duplex.

**Specificity.** RNAi-mediated silencing of gene expression can be highly specific, as evidenced by selective silencing of alleles containing single nucleotide polymorphisms<sup>18</sup>. Nevertheless, siRNAs also recognize and interfere with the expression of mRNAs that share partial homology with the target mRNA. *In vitro* transcriptional profiling has shown that siRNAs can alter the mRNA levels of off-target genes in addition to the intended target gene but usually the changes are less than threefold<sup>19–21</sup>. An accurate assessment of off-target silencing would require detailed proteomic analyses to identify these genes. The tools for unbiased analysis of protein expression are less developed at present and have not been applied to examine off-target silencing. Not surprisingly, many of the off-target genes that have reduced mRNA levels contain regions that are complementary to one of the two strands in the siRNA duplex<sup>19–21</sup>. Complementarity between the 5'-end of the guide strand and the mRNA is key to off-target silencing with the critical nucleotides being in positions



**Figure 2 | Turning siRNA into drugs.** This three-step process begins with *in silico* design and *in vitro* screening of target siRNAs, is followed by incorporating stabilizing chemical modifications on lead siRNAs as required, and ends with the selection and *in vivo* evaluation of delivery technologies that are appropriate for the target cell type/organ and the disease setting. A schematic illustration of the important features of siRNA structure (two base pair overhangs, seed region and mRNA cleavage site) is shown. An example of an optimized siRNA molecule that incorporates chemical modifications to increase nuclease stability and to minimize off-targeting is shown below. The phosphorothioate (P=S) and 2'-base sugar modifications (green circles) are illustrative of exonuclease and endonuclease stabilizing chemistries, respectively; other chemistries that confer similar properties on an siRNA duplex also exist<sup>39</sup>. The passenger strand and the guide strand are represented in blue and orange, respectively.

two to eight (from the 5'-end of the guide strand) or the so-called 'seed region' for microRNA recognition of endogenous target mRNAs<sup>22,23</sup> (FIG. 2). Accordingly, one approach for minimizing off-target effects is the careful design of siRNA to minimize exact seed pairings, although seed regions of seven nucleotides are very short in length. Alternatively, chemical modifications of riboses in the guide strand can suppress most off-target effects, while maintaining target-mRNA silencing<sup>24,25</sup>. In particular, a single 2'-O-methyl modification at nucleotide 2 suppresses off-target silencing without significantly interfering with on-target silencing. Thus, bioinformatics design and/or position-specific, sequence-independent chemical modifications can be used to reduce sequence-related off-target effects while maintaining efficient target-mRNA silencing.

siRNA can also induce potential unwanted effects by activating the innate immune response, setting off defence systems usually used to combat viruses. One pathway involves recognition of dsRNAs by the serine/threonine protein kinase PKR (reviewed in REF. 26). This pathway is efficiently triggered only by dsRNAs that are more than 30 nucleotides long. At higher concentrations, however, smaller siRNAs may be able to activate this pathway, resulting in global translational blockade and cell death. Perhaps of more concern is the potential to activate Toll-like receptors (TLRs), especially the dsRNA receptor TLR7 in plasmacytoid

dendritic cells, to trigger the production of type I interferons and pro-inflammatory cytokines, and induce nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation<sup>27</sup>. In much the same way that certain CpG motifs in antisense oligonucleotides mediate TLR9 immunostimulation, interferon induction by some siRNAs occurs principally through TLR7, which is largely found in endosomes of immune antigen-presenting cells. Fortunately, TLR7 binding is sequence specific, favouring GU-rich sequences, and can be avoided by choosing sequences that are not recognized by this receptor. Additional work is required to identify immunostimulatory motifs and determine whether other receptors might also be involved. Several approaches exist to circumvent the immunostimulatory properties of certain siRNA duplexes. Candidate duplexes can be transfected into plasmacytoid dendritic cells to test whether they induce interferon expression<sup>27,28</sup>; those that do can be discarded. Moreover, the presence of 2'-O-methyl modifications within the siRNA duplex can be used to abrogate TLR7 binding and abolish immunostimulatory activity<sup>27,29</sup>. These modifications also reduce sequence-dependent off-target silencing and may be particularly beneficial in enhancing siRNA target specificity. Potentially, TLR7 signalling could also be evaded by using siRNA delivery strategies that avoid the cell types responsible for immune stimulation or that transport the siRNA directly to the cytoplasm, thereby avoiding TLR activation in the endosome.

**Stability.** Naked siRNAs are degraded in human plasma with a half-life of minutes<sup>30,31</sup>. To convert siRNAs into optimized drugs, the chemical modifications that prolong siRNA half-life without jeopardizing biological activity have been investigated in detail. The starting point for these efforts has been chemistries that are already in use with antisense oligonucleotide and aptamer therapeutics. For instance, the introduction of a phosphorothioate (P=S) backbone linkage at the 3'-end protects against exonuclease degradation, and 2'-sugar modification (such as 2'-O-methyl or 2'-fluoro) provides endonuclease resistance<sup>30-33</sup> (FIG. 2). With respect to maintaining RNAi silencing activity, exonuclease-stabilizing modifications are well tolerated. Introduction of internal sugar modifications to protect against endonucleases is also generally tolerated but can depend on the location of the modification within the duplex; the sense strand being more amenable to modification than the antisense strand. Nevertheless, using simple, well-described modifications such as P=S, 2'-O-methyl and 2'-fluoro it is possible, in most instances, to stabilize an siRNA duplex while maintaining silencing activity. The minimal modifications required to stabilize a particular siRNA duplex can be identified by studying its degradation fragments in the plasma, thereby avoiding the toxicities associated with certain oligonucleotide chemistries and minimizing changes that could reduce activity.

Information about siRNA stability in other body fluids is not published and needs to be considered depending on the application. When siRNAs are formulated into different carrier systems for *in vivo* delivery they may be protected from nuclease digestion and last longer than naked siRNAs. Improved nuclease-stability is especially important for siRNA duplexes that are exposed to nuclease-rich environments (such as blood) and that are formulated using excipients that do not themselves confer additional nuclease protection. As might be expected in these situations, nuclease-stabilized siRNAs show improved pharmacokinetic properties *in vivo*<sup>34</sup>. In other situations, when delivering siRNA directly to less nuclease-rich sites, such as the lung, or when delivering siRNA in conjunction with delivery agents such as liposomes, the degree of nuclease stabilization can be reduced significantly. Although the ability of an siRNA duplex to reach its target cell intact is vitally important, whether nuclease protection confers a measurable benefit once an siRNA is inside

the cell remains to be determined. *In vitro* comparisons of naked siRNA versus fully stabilized siRNA do not reveal significant differences in longevity of mRNA silencing<sup>30</sup>, but these studies have typically been carried out using rapidly dividing cells in which dilution due to cell division, and not intracellular siRNA half-life, governs the duration of gene silencing<sup>35</sup>. However, the durability of silencing in non-dividing cells *in vitro* and *in vivo* — lasting for weeks — suggests that stabilization might not be necessary within the cell. Recently developed fluorescence resonance energy transfer techniques (FRET) for studying siRNAs<sup>36</sup> should be useful for evaluating whether nuclease-stabilization enhances intracellular activity.

**Therapeutic considerations.** In designing siRNAs for therapeutic purposes there are other considerations beyond finding an active target sequence. If possible, it is desirable to identify target sequences that are conserved across all the relevant species used in safety and efficacy studies, thus enabling the development of a single drug candidate from the research stage through to clinical studies. Much progress has been made in understanding what attributes identify an active and stable siRNA *in vitro*, but much less is known about how well those attributes translate into *in vivo* activity. Most of our knowledge on siRNA specificity is based only on *in vitro* data. Furthermore, the range of off-target genes identified in tissue culture can differ dramatically depending on the transfection method used to introduce siRNAs into cells and the mRNA expression profile of the target cell<sup>37</sup>. Likewise, induction of innate immune responses by certain siRNAs is cell-type specific<sup>14</sup>. Because of the uncertainty about *in vivo* properties, the practical and prudent approach is to identify several lead candidate siRNAs that are suitable for subsequent *in vivo* study by selecting from a library of tested siRNA duplexes those that are most potent, specific and stable to endogenous nucleases, but that lack immunostimulatory activity.

**In vivo delivery**

Effective delivery is the most challenging hurdle remaining in the development of RNAi as a broad therapeutic platform. Animal studies using siRNA have either used no additional formulation (naked siRNA) or have delivered siRNA formulated as conjugates, liposome/lipoplexes or as complexes with peptides, polymers or antibodies (FIG. 3). The challenges of local, direct delivery have been elucidated for some tissue sites, and

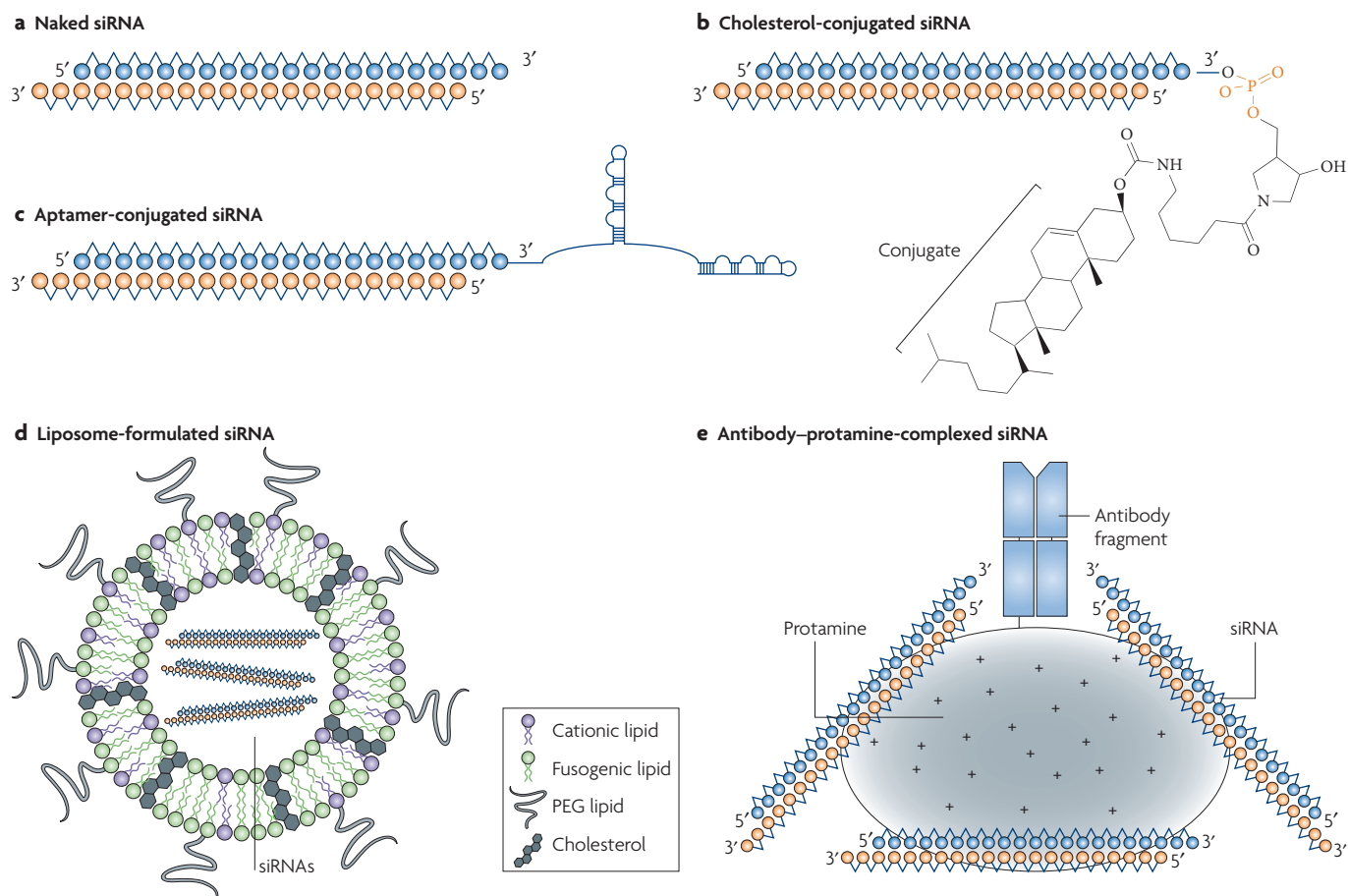
locally delivered siRNAs are being tested in ongoing clinical studies in the eye for AMD and in the lung for RSV infection (see the clinical trials section for details). Local delivery or ‘direct RNAi’ has the advantage that, as for any drug, the dose of siRNA required for efficacy is substantially lower when injected into or administered at, or near, the target tissue. Direct delivery might also reduce any undesired systemic side effects. Systemic delivery of siRNA to target tissues deep within the body remains challenging although there have been some recent successes in animal models. This section will review different siRNA delivery approaches; examples of *in vivo* success using siRNA are summarized in TABLE 1. Several recent publications offer more comprehensive reviews on this topic<sup>33,38,39</sup>.

**Naked siRNA.** Many reports describing success with RNAi *in vivo* involve direct delivery of naked siRNA to tissues such as the eye, lung and central nervous system (CNS) (TABLE 1). It is not understood why certain cells, but not others, can directly take up siRNAs into the cytoplasm where the RNAi machinery operates. As used here, the term ‘naked’ siRNA refers to the delivery of siRNA (unmodified or modified) in saline or other simple excipients, such as 5% dextrose (D5W). The ease of formulation and administration using direct delivery of naked siRNA to tissues make this an attractive therapeutic approach. Not surprisingly, the initial development of RNAi therapeutics has focused on disease targets and clinical indications (such as AMD and RSV infection) that allow for direct administration of siRNA to the diseased organ.

**Ocular delivery.** Proof-of-concept of siRNA efficacy in the eye was first demonstrated in animal models of ocular neovascularization and scarring using both saline and lipid-based formulations<sup>40–43</sup>. Lipid-formulated vascular endothelial growth factor (VEGF) siRNA reduced laser-induced choroidal neovascularization in a mouse model of AMD<sup>40</sup>. In the same scarring model in non-human primates intravitreal injection of saline-formulated VEGF siRNA was well tolerated and efficacious<sup>41</sup>. Intravitreal injection of saline-formulated siRNA targeting VEGF receptor-1 was also effective in reducing the area of ocular neovascularization in two different mouse models<sup>42</sup>. These encouraging studies laid the ground work for clinical testing of siRNA targeting the VEGF pathway in AMD.

**Respiratory delivery.** Intranasal or orotracheal administration of formulated siRNA results in effective gene silencing in the lung and therapeutic benefit in a number of pulmonary disease models. Most of these studies used naked siRNA either in saline or with excipients such as D5W or lung surfactants. Formulating the siRNA with a transfection lipid or another specialized delivery carrier is sometimes dispensable. However, the required dose or delivery to certain cell types in the lung for some indications might be improved by incorporating the siRNA into carriers. In one study, intranasal instillation of siRNAs (either unformulated or complexed with the transfection agent Transit-TKO) directed against viral genes reduced the viral load of RSV and parainfluenza virus, two relevant pathogens in paediatric and immunocompromised patients, by more than three orders of magnitude, using doses of siRNA as low as 70 µg per animal with no observed adverse events<sup>44</sup>. Other pathological features of RSV infection such as elevated leukotriene levels, pulmonary inflammation and clinical signs of respiratory distress were also reduced after siRNA administration. Protection could be achieved in both prophylaxis and treatment paradigms. Similarly, siRNA formulated in D5W administered intranasally in a non-human primate model of severe acute respiratory syndrome (SARS) corona virus infection significantly reduced interstitial infiltrates and pathological changes, and inhibited viral replication in the lung<sup>45</sup>. These studies clearly demonstrate the potential of RNAi therapeutics to treat viral respiratory infection.

Gene silencing by locally delivered siRNAs in the lung has also been demonstrated in studies designed to identify the key molecules that are responsible for lung injury in various models of acute lung injury (ALI). Intranasal administration of siRNAs targeting haeme oxygenase 1 (HO1, also known as HMOX1) resulted in enhanced apoptosis in an ischaemia–reperfusion (I-R) mouse model, demonstrating that induction of this gene is protective<sup>46</sup>. While I-R induced HO1 expression in a number of organs, intranasally administered siRNA only silenced gene expression in the lung. Another study in a haemorrhage-induced and septic challenge model of ALI showed that siRNA targeting keratinocyte-derived chemokine (KC) and macrophage-inflammatory protein 2 (MIP2, also known as CXCL2) administered intratracheally decreased lung KC and MIP2 mRNA by approximately 50%, reducing local interleukin 6 (IL-6) concentrations and, in the case of MIP2 siRNA, reducing neutrophil influx, interstitial oedema and lung



**Figure 3 | Delivery of small interfering RNAs.** Different strategies have been used to deliver and achieve RNAi-mediated silencing *in vivo*. **a** | Direct injection of naked siRNA (unmodified or chemically modified) has proven efficacious in multiple contexts of ocular, respiratory and central nervous system disease<sup>40–57</sup>. **b** | Direct conjugation of siRNA to a natural ligand such as cholesterol has demonstrated *in vivo* silencing in hepatocytes following systemic administration<sup>59</sup>. **c** | Aptamer–siRNA conjugates silence target genes in tumour cells following local injection in a tumour xenograft model<sup>64</sup>. **d** | Liposome–formulated delivery of siRNA has been used to silence multiple targets following systemic administration<sup>69–71,73</sup>. The composition of a stable nucleic acid–lipid particle (SNALP) is shown. Cationic lipids aid

formulation, cellular uptake and endosomal release. Fusogenic lipids also function in endosomal release. Polyethylene glycosylated (PEG) lipids stabilize the formulation, regulate fusogenicity and shield surface charges. Cholesterol also helps to stabilize the formulation. Other cationic liposome formulations exist which may or may not contain a fusogenic or PEG lipid. The ratios of different components can vary between formulations. **e** | Antibody–protamine fusion proteins have been used to non-covalently bind siRNAs through charge interactions and to deliver siRNA specifically to cells that express the surface receptor that is recognized by the antibody<sup>101,102</sup>. The passenger strand and the guide strand are represented in blue and orange, respectively.

pathology<sup>47</sup>. Silencing with Fas-siRNAs in the same model suppressed the induction of *Fas* and ameliorated pulmonary apoptosis and inflammation<sup>48</sup>. In hyperoxia-induced ALL, intranasal administration of an angiotensin 2 (ANGPT2) siRNA in saline dramatically ameliorated oxidant injury, cell death, inflammation, vascular permeability and mortality<sup>49</sup>. In bleomycin-induced lung fibrosis, intranasal instillation of siRNA targeting Discoidin Domain Receptor 1 (DDR1) reduced DDR1 protein in the lung by 60–70% and attenuated lung inflammatory cell infiltrates and cytokine production<sup>50</sup>. These studies clearly demonstrate the potential for direct instillation of naked siRNA to silence endogenous genes in the lung and treat pulmonary disease.

**CNS delivery.** Direct instillation of siRNA in saline has also been used in the CNS to validate disease targets *in vivo*. Direct administration of saline-formulated siRNA by intracerebroventricular, intrathecal or intraparenchymal infusion resulted in silencing of specific neuronal mRNA targets in multiple regions of the peripheral and central nervous system<sup>51–54</sup>. Although the naked unformulated siRNA dose that is typically required for target silencing in these rodent studies is high (~0.5 mg per day) and siRNA uptake is uneven, polymer or lipid-based delivery systems facilitate cellular uptake; effective *in vivo* doses are reduced to ~50 µg with these formulations<sup>55–57</sup>.

**Conjugation.** Covalent conjugation of siRNAs to a targeting molecule has been used to deliver siRNA into cells and can be used, in principle, for cell-type-specific targeting. Through conjugation, the therapeutic can be fixed as a single compound, reducing the complexity from a chemical, manufacturing and regulatory perspective. Conjugation is especially attractive for siRNA-based drugs as only one of the strands in the duplex is active and conjugates can be attached to the sense or passenger strand without disrupting the activity of the active antisense strand. Conjugates can be placed on either the 5'- or 3'-end of the sense strand, although in some instances they can also be tolerated on the 3'-end of the

Table 1 | Selected examples of in vivo efficacy using siRNA in disease models

Target	Formulation	Route	Model	Refs
<b>Ocular</b>				
VEGF	Lipoplex	Subretinal	Laser-induced CNV	40
VEGF	Saline	Intravitreal	Laser-induced CNV	41
VEGF R1	Saline	Intravitreal	Laser-induced CNV; ROP	42
TGFβ R2	Lipoplex	Subconjunctival	Subconjunctival scarring	43
<b>Pulmonary</b>				
RSV-P, PIV-P	Saline or lipoplex	Intranasal	RSV infection; PIV infection	44
SARS	D5W or surfactant	Intranasal	SARS infection	45
HMOX1	Saline	Intranasal	Hyperoxic acute lung injury	46
KC, MIP2, Fas	Saline	Intranasal	Septic acute lung injury	47,48
ANGPT2	Saline	Intranasal	Hyperoxic acute lung injury	49
DDR1	Saline	Intranasal	Bleomycin-induced fibrosis	50
Caveolin-1	Liposomes	Intravenous	Vascular permeability	73
Influenza A – NP, PA	PEI	Intravenous	Influenza virus infection	82
GFP	Chitosan nanoparticle	Intranasal	GFP transgenic	89
<b>Nervous System</b>				
AGRP	Saline	Stereotactic injection	Metabolic alterations	51
Dopamine transporter	Saline	Intraventricular	Hyperlocomotor response	52
Serotonin transporter	Saline	Intraventricular	Behavioural response	53
P2X3	Saline	Intrathecal	Chronic neuropathic pain	54
DOR	Lipoplex	Intrathecal	DELTA-induced nociception	55
NMDAR subunit NR2B	PEI	Intrathecal	Formalin-induced nociception	56
JEV and WNV-envelope	Lipoplex	Intracranial	JEV- and WNV-induced encephalitis	57
<b>Liver</b>				
ApoB	Cholesterol conjugate	Intravenous	Dyslipidaemias	59
ApoB	Liposome	Intravenous	Dyslipidaemias	69
HBV	Liposome	Intravenous	Plasmid-based HBV replication	70
Ebola L gene	PEI or Liposome	Intraperitoneal	Ebola virus infection	71
<b>Tumours</b>				
EWS-FLI1	Tf-targeted nanoparticle	Intravenous	Ewing's sarcoma model	61
VEGF R2	RGD peptide-targeted PEI	Intravenous	Subcutaneous tumour xenograft	63
PLK1, BCL2	PSMA aptamer	Intratatumoral	Subcutaneous tumour xenograft	64
HPV-E6	Lipoplex	Intratatumoral or intraperitoneal	Subcutaneous tumour xenograft	74
Pleotrophin	PEI	Intraperitoneal or subcutaneous	Subcutaneous and glioblastoma tumour xenografts	83
HER2	PEI	Intraperitoneal	Subcutaneous tumour xenograft	84
RhoA	Chitosan nanoparticle	Intravenous	Subcutaneous tumour xenograft	90
VEGF	Atelocollagen	Intravenous	Subcutaneous tumour xenograft	92
VEGF	Cholesteryl oligoarginine	Intratatumoral	Subcutaneous tumour xenograft	99
C-Myc, MDM2, VEGF	HIV envelope protamine fusion	Intravenous	Subcutaneous tumour xenograft	101
<b>Other Organs</b>				
TNF	Lipoplex	Intravenous	Collagen-induced arthritis	72
HSV2 – UL27, UL29	Lipoplex	Intravaginal	Herpes simplex 2 virus infection	75
TNF	Lipoplex	Intrarectal	DSS-induced colitis	76

AGRP, agouti-related peptide; ANGPT2, angiopoietin 2; ApoB, apolipoprotein B; CNV, choroidal neovascularization; D5W, 5% dextrose; DELTA, D-Ala Glu deltorphin II; DDR1, Discoidin domain receptor 1; DOR, delta opioid receptor; DSS, dextran sodium sulfate; GFP, green fluorescent protein; HBV, hepatitis B virus; HER2, human epidermal growth factor receptor 2; HMOX1, haeme oxygenase 1; HPV, human papilloma virus; JEV, Japanese encephalitis virus; KC, keratinocyte-derived chemokine; MIP2, macrophage-inflammatory protein 2; NMDA R, N-methyl-D-aspartate (NMDA) receptor; NP, nucleoprotein gene; PA, polymerase gene, acidic; PEI, polyethylenimine; PIV, parainfluenza virus; PIV-P, PIV phosphoprotein gene; PSMA, prostate-specific membrane antigen; RGD, Arg-Gly-Asp; ROP, retinopathy of prematurity; RSV, respiratory syncytial virus; RSV-P, RSV phosphoprotein gene; SARS, severe acute respiratory distress syndrome; Tf, transferrin; TGFβ R2, transforming growth factor receptor 2; TNF, tumour necrosis factor α; VEGF, vascular endothelial growth factor; VEGF R1/2, VEGF receptor 1/2; WNV, West Nile virus.

antisense strand. To date, siRNA conjugates have been made using lipophilic molecules, proteins, peptides and aptamers.

Conjugation to cholesterol, previously used to deliver antisense oligonucleotides to the liver<sup>58</sup>, has been adapted to achieve effective *in vivo* silencing by siRNAs<sup>59</sup>. Intravenous injection of siRNA duplexes conjugated to cholesterol at the 5'-end of the passenger strand targeting apolipoprotein B (ApoB) in mice silenced ApoB mRNA by ~55% in the liver and ~70% in the jejunum, the two main sites of ApoB expression. Concomitantly, ApoB plasma protein dropped by ~70% and serum cholesterol fell by 35–40%, consistent with the biological function of ApoB in binding cholesterol. Cholesterol conjugation was critical for improved pharmacokinetics and *in vivo* cellular uptake as the unconjugated ApoB siRNA was rapidly cleared and inactive. However, these experiments required a relatively high dose (50 mg per kg) of siRNA. Cholesterol conjugation has been reported to prolong circulating half-life by incorporating the cholesterol–siRNA conjugate into circulating lipoprotein particles that are resistant to filtration by the kidney and may also provide protection from plasma nucleases. The siRNA-containing lipoprotein complexes are endocytosed through cholesterol receptors present on all cells (M. Stoffel, personal communication).

Other natural ligands have been directly conjugated to siRNA duplexes and shown to facilitate delivery, at least *in vitro*. These include membrane permeant peptides such as penetratin and transportan<sup>60</sup>, but robust evidence for improved cellular delivery by these conjugates is still lacking. Although certain natural ligands such as transferrin, folate and RGD (Arg-Gly-Asp) peptides have been successfully used as targeting agents for siRNA when formulated into nanoparticles<sup>61–63</sup>, these have yet to be thoroughly investigated as direct siRNA conjugates. Finally, siRNA conjugation to small molecules that bind to cell-surface receptors that are present on a limited number of cells should, in theory, enhance drug specificity and reduce the required drug concentration. Evidence for the *in vivo* use of small-molecule siRNA conjugates has so far not been published, and it is important to remember that these small molecules might not have the benefit of natural ligand conjugates, such as cholesterol, in facilitating siRNA incorporation into larger plasma protein complexes that evade rapid renal excretion and elimination.

siRNAs can also be conjugated to RNA aptamers for delivery to specific cell types.

This approach has the advantage that the therapeutic can be composed entirely of RNA. Recently, *in vitro* and *in vivo* proof-of-concept has been shown using aptamers to prostate-specific membrane antigen (PSMA), a cell-surface receptor overexpressed in prostate cancer cells and tumour vascular endothelium<sup>64,65</sup>. PSMA aptamers, either directly linked to siRNA<sup>64</sup> or conjugated through a modular streptavidin bridge<sup>65</sup>, promote specific cellular uptake and RNAi-mediated silencing of target mRNA *in vitro*. siRNAs directed against survival genes (*PLK1* and *BCL2*) and directly linked to PSMA aptamers were internalized by cells and resulted in RNAi-mediated target-mRNA silencing and cell death *in vivo*<sup>64</sup>. Short aptamers (25–35 bases long) that bind to a wide variety of targets with high affinity have been described<sup>66</sup>. It should be possible to design siRNA-aptamer chimeras with longer strands (45–55 bases) that are within the range of technical and commercial feasibility for synthesis, but longer molecules may prove difficult to synthesize on a commercial scale. However, although short aptamer conjugates work for local delivery *in vivo*, they can be rapidly cleared by the kidney and have a half-life that is too short for systemic therapy unless they are incorporated into larger particles.

**Liposomes and lipoplexes.** Many drugs have been formulated into liposomes to improve their pharmacokinetic properties and decrease toxicity. Liposomes, vesicles with an aqueous compartment enclosed in a phospholipid bilayer, can fuse with cell membranes and enhance drug delivery into cells. Polar drugs can be entrapped in their aqueous centre. When lipids complex with nucleic acids to form amorphous particles they are known as lipoplexes. Lipoplexes are formed by mixing siRNAs with most commercial transfection agents, such as Lipofectamine 2000 and Transit-TKO. Liposomes developed for therapeutic delivery of siRNAs are multi-component nanoparticles, typically composed of multiple lipids, including a cationic and/or a fusogenic lipid, cholesterol and a polyethylene glycosylated lipid. Each of these components has an important role in the fusogenicity and pharmacokinetic properties of the liposome (FIG. 3d; for review see REFS 67–68).

Both liposomes and lipoplexes have been extensively used to deliver siRNA *in vitro* and *in vivo*. *In vitro* transfection of siRNA using lipid-based delivery agents is a routine laboratory procedure. However, some primary cell types, such as lymphocytes,

are resistant to lipid-mediated transfection. Significant success has also been demonstrated with both local and systemic administration of siRNA using liposomes and lipoplexes. Intravenous injection of siRNA encapsulated in stable nucleic acid–lipid particles (SNALPs<sup>28</sup>) silenced ApoB dramatically in both mice and non-human primates<sup>69</sup>. Importantly, a single intravenous dose of 2.5 mg per kg of SNALP-formulated siRNA in cynomolgus monkeys reduced ApoB mRNA in the liver by more than 90%. Consequently, serum cholesterol and low-density lipoproteins were reduced by more than 65% and 85%, respectively. Moreover, following a single intravenous dose of 2.5 mg per kg of SNALP-formulated siRNA, gene silencing lasted for at least 11 days. Treatment with siRNA in liposomes was fairly well tolerated in these experiments, with transient increases in liver enzymes seen at the highest dose. The same SNALP formulations also effectively delivered siRNA to the liver in animal models of hepatitis B and Ebola virus infection<sup>70,71</sup>. Whether these SNALPs can be used for delivering siRNA to organs other than the liver is uncertain. Systemic administration of other liposomal formulations and lipoplexes have resulted in the delivery of siRNA to other tissues. Intravenous delivery of a cationic lipoplex containing tumour necrosis factor (TNF) siRNA specifically inhibited TNF production in knee joints and alleviated disease in a mouse model of rheumatoid arthritis<sup>72</sup>, whereas caveolin-1 siRNA formulated in liposomes reduced target expression by 90% in mouse lung endothelia with the expected physiological effects<sup>73</sup>. In summary, use of lipid-based formulations for systemic delivery of siRNA, especially to the liver, represents one of the most promising near-term opportunities for the development of RNAi therapeutics.

Locally injected liposomes and lipoplexes have also delivered siRNA effectively to target cells in the eye and nervous system, and to tumours. Subretinal injection of VEGF siRNA formulated in Transit-TKO reduced choroidal neovascularization in a laser-induced injury mouse model<sup>40</sup>, and subconjunctival injection of transforming growth factor  $\beta$  receptor II siRNA in Transit-TKO suppressed inflammation and fibrosis in a mouse subconjunctival scarring model<sup>43</sup>. Intracranial delivery of lipid-complexed siRNA targeting viral genes protected mice against fatal Japanese encephalitis virus and West Nile virus encephalitis<sup>57</sup>. Similarly, intrathecal administration of cationic lipid formulated delta opioid receptor (DOR)

siRNA facilitated delivery and specific target-gene silencing in the spinal cord and dorsal root, blocking antinociception by a DOR selective agonist<sup>55</sup>. Numerous publications have reported successful antitumoural effects following either direct or systemic tumoural injection of lipid-formulated siRNA (for reviews see REFS 33,38,39). In one recent example, either intratumoural or intraperitoneal injection of formulated siRNA directed against the human papilloma virus E6 oncogene inhibited tumour growth, suppressed E6 expression and induced tumour apoptosis in subcutaneous cervical cancer xenografts<sup>74</sup>. The usefulness of lipid-based delivery systems in many of these direct RNAi applications must be assessed in a target-cell-specific and disease-specific manner. For the eye, lung and nervous system there are some examples of siRNA being successfully delivered without formulating agents. However, there are other examples in which formulations were critical.

An area of increasing interest is direct application of lipoplexed siRNA to mucosal surfaces such as the vagina and the intestine. Intravaginal delivery of lipid-complexed siRNA directed against herpes simplex 2 virus protected mice when administered before and/or after lethal herpes virus challenge<sup>75</sup>. Intravaginal administration of siRNA complexed with Lipofectamine 2000, but not naked siRNA, also specifically silenced endogenous genes in the genital tract<sup>76</sup>. Intrarectal administration of TNF siRNA in Lipofectamine also reduced TNF levels and attenuated colonic inflammation in the descending colon<sup>76</sup>. Intravaginal and intracolonic administration of siRNA using these lipid-based delivery systems was well tolerated in mice, lasted for at least a week and did not cause inflammation or activate an interferon response<sup>75,76</sup>. Because of the relatively easy access to mucosal sites, the large number of viral infections that enter the body through mucosal portals, the general tolerability of siRNA-lipid formulations and robust *in vivo* delivery, mucosal administration of lipid-formulated siRNA will be a fertile area for developing future RNAi therapeutics.

**Peptides and polymers.** siRNA can also be complexed with cationic peptides and polymers by ionic interactions with their negatively charged phosphate backbones to form stable nanoparticles<sup>77</sup>. Strategies are needed to prevent aggregation and control particle size in a reproducible manner. This is most often done by incorporating molecules such as polyethylene glycol (PEG)

that help to stabilize the particle and prevent aggregation. PEG groups also improve the pharmacokinetic profile of these particles by shielding positive charges on the surface that will stick to negatively charged cell membranes and result in rapid clearance from the circulation.

Another area of particle engineering, applicable to both liposomes and polymeric nanoparticles, involves the use of targeted nanoparticles to achieve robust *in vivo* targeting to particular cell types. Molecules such as transferrin<sup>61</sup>, folate<sup>62</sup> and RGD peptide<sup>63</sup> can be introduced into particle-based delivery vehicles to aid siRNA targeting to cells bearing the natural receptor. Other targeting groups including antibodies or sugars, such as galactose and mannose, have also been proposed to target siRNA to specific tissues<sup>78</sup>. Beyond improving particle targeting *in vivo*, another important area of research involves increasing cytoplasmic delivery of siRNA. Several pH-triggered polymers that facilitate endosomal escape and that could be incorporated into nanoparticles have been identified<sup>68,79</sup>; data already exist to show that one of these may improve RNAi-mediated silencing *in vitro*<sup>80</sup>. Targeted delivery and improved cytoplasmic delivery of nanoparticles is a promising field of active research.

One of the most widely studied polymers for delivery of nucleic acids is polyethylenimine (PEI). PEI polymers are synthetic, linear or branched structures with high cationic charge densities. The cationic polyplexes formed by PEI with siRNA are thought to interact with the cell surface electrostatically and are endocytosed into cells where they disrupt the low endosomal pH. Endosomal escape is proposed to occur by a 'proton sponge' effect whereby PEI enhances the influx of protons and water leading to endosomal swelling and to the osmotic release of the polyplexes into the cytoplasm<sup>81</sup>. PEI-siRNA complexes have been reported to show therapeutic benefit *in vivo* in a number of disease models. PEI-complexed with influenza siRNA demonstrated antiviral effects in infected mice<sup>82</sup>, whereas similar targeting of the Ebola L gene resulted in partial protection against lethal Ebola infection in guinea pigs<sup>71</sup>. PEI-siRNA complexes targeting pleiotrophin<sup>83</sup>, VEGF<sup>63</sup> and human epidermal growth factor receptor 2 (HER2, also known as ERBB2) (REF. 84) have been shown to have antitumour activity. Local injection of PEI-siRNA polyplexes targeting the *N*-methyl-D-aspartate (NMDA) receptor subunit NR2B significantly attenuated formalin-induced nociception in rats<sup>56</sup>.

A significant concern about using PEI as a therapeutic delivery vehicle is the extreme toxicity seen at higher doses. To expand the safety margin several groups are optimizing the physical structure of PEI to improve *in vivo* siRNA delivery<sup>85-87</sup>. Other synthetic polycations, consisting of histidine and polylysine residues, have also been evaluated for delivery of siRNA and seem to have improved *in vitro* efficacy compared with PEI<sup>88</sup>.

Three other polymer approaches with promising *in vivo* siRNA data are chitosan, cyclodextrin, and atelocollagen (protease-treated collagen) nanoparticles. Chitosan is a well-tolerated natural biodegradable polymer that forms cationic complexes with nucleic acids. Effective *in vivo* RNAi delivery was achieved by chitosan-siRNA complexes both in lung epithelial cells of transgenic enhanced green fluorescent protein (EGFP) mice after intranasal administration<sup>89</sup> and in subcutaneously implanted breast cancer cells after intravenous administration<sup>90</sup>. Transferrin-targeted cyclodextrin-containing polycation nanoparticles silenced the oncogene *EWS-FLI1* in transferrin receptor-expressing Ewing's sarcoma tumour cells<sup>61</sup> and siRNA formulated in these nanoparticles was well tolerated in non-human primates<sup>91</sup>. Systemic administration of atelocollagen-siRNA complexes had marked effects on subcutaneous tumour xenografts as well as on bone metastases<sup>92,93</sup>. Other polymer approaches, based on polyamidoamine (PAMAM) dendrimers and poly(lactic-co-glycolic acid) (PLGA) nanoparticles, are also being investigated as vehicles for siRNA delivery<sup>94,95</sup>, although *in vivo* evidence for RNAi-mediated silencing using these approaches remains to be demonstrated.

Cationic peptides can also deliver oligonucleotides into cells<sup>67,96</sup>. Several peptide-based gene delivery systems, formed with peptides such as MPG (27mer peptide)<sup>97</sup>, penetratin<sup>98</sup> and cholesteryl oligo-D-arginine (Chol-R9)<sup>99</sup>, promote the uptake of non-covalently bound siRNAs *in vitro*. Local *in vivo* administration of chol-R9-complexed VEGF siRNA led to tumour regression in a mouse model<sup>99</sup>. Peptide-based approaches can also be coupled with other delivery systems, such as liposomes, to enable a more targeted delivery of oligonucleotides<sup>63,67,68,100</sup>.

**Antibodies.** Antibodies can also be used for specific targeted delivery of siRNA. A protamine-antibody fusion protein delivered non-covalently bound siRNA to HIV-envelope-expressing B16 melanoma cells or HIV-infected primary CD4 T cells *in vitro* with high efficiency<sup>101</sup>. In this



approach, a protamine fragment that binds to the oligonucleotide and the Fab fragment of an HIV envelope antibody mediates receptor-specific binding to cells expressing the HIV envelope protein. These siRNA-antibody-protamine complexes, administered either intra-tumourally or intravenously, specifically delivered siRNA to a subcutaneous tumour *in vivo* and retarded tumour growth. A single-chain antibody to the breast cancer receptor HER2 fused to protamine also selectively delivered siRNAs to breast cancer cells bearing the receptor. Recently, fusion proteins that target all human white blood cells or just activated leukocytes using conformation sensitive, single chain antibodies to the integrin LFA-1, have revealed the exquisite selectivity of this targeting strategy both *in vitro* and *in vivo*<sup>102</sup>. These studies demonstrate the potential for antibodies to direct siRNA selectively into cells *in vivo*.

### Clinical trials

RNAi has rapidly advanced from research discovery to clinical trials. Three different RNAi therapeutics are currently under clinical investigation, with several more poised to enter trials soon. Initial trials have focused on well-validated therapeutic targets, such as the VEGF pathway for the wet form of AMD and on the RSV genome, for the treatment of RSV infection. Ophthalmic indications have historically been attractive for oligonucleotide-based therapeutics. The only approved oligonucleotide-based drugs are used in the eye — Vitravene<sup>®</sup> to treat CMV retinitis<sup>103</sup> and Macugen<sup>®</sup> for AMD<sup>104</sup>. Direct injection into the vitreal cavity efficiently targets oligonucleotide drugs to the retina. Another advantage of the ocular compartment is that it is relatively free of nucleases compared with serum, permitting unmodified siRNA to be used in the vitreal cavity. However, exonuclease stabilizing chemistries can dramatically improve the persistence of intact siRNA following intravitreal injection (Alnylam, unpublished results). The ease of intraocular drug delivery combined with the clinical validation of VEGF as a therapeutic target for proliferative vascular retinopathies has led to the rapid development of several RNAi therapeutics.

Intravitreal injection of Cand5, an unmodified siRNA that targets all VEGF-A spliced isoforms, has completed a phase II trial in patients with serious progressive wet AMD and has been reported to provide dose-related benefits against several endpoints, including near vision and lesion size (Acuity Pharmaceuticals, press release). Cand5 is also being tested for efficacy against diabetic

macular oedema in a phase II trial that began in early 2006 (Acuity Pharmaceuticals, press release). Sirna-027, a chemically modified siRNA that targets VEGF receptor 1, completed phase I trials in patients with wet AMD and was reported to be well tolerated. It was also reported to stabilize or improve visual acuity in a subset of patients (Sirna Pharmaceuticals, press release).

There are many advantages to delivering siRNA therapeutics directly to the lungs for targeting lung epithelial cells. Lung epithelial cells are capable of naked siRNA uptake and RNAi mediated silencing<sup>44,46,49</sup>. Moreover the local lung environment is also relatively nuclease-free compared with serum. Delivery to the lung by inhalation is non-invasive and directly targets the tissue epithelium; this improves drug concentrations at the target tissue without first-pass metabolism, reduces drug dosing and decreases the likelihood of systemic side effects. In many instances naked siRNA in saline seems to be effective, but other approaches to optimize lung delivery may need to be developed for different indications.

The first pulmonary studies are directed at treating RSV, a serious neonatal respiratory infection. Two phase I intranasal trials with ALN-RSV01, an siRNA targeting the viral nucleocapsid gene, have been completed in over 100 healthy adult volunteers. ALN-RSV01 was found to be safe and well tolerated (Alnylam Pharmaceuticals, press release). Clinical development of ALN-RSV01 is progressing; experimental infection studies are ongoing.

### Summary

RNAi has advanced from research discovery to clinical trials with lightning speed — from the Nobel-prize winning discovery in 1998 showing that long dsRNA could silence gene expression in *Caenorhabditis elegans*<sup>1</sup>, to the publication in 2001 showing that short synthetic siRNA could induce RNAi in mammalian cells<sup>2</sup>, to the present day when three different RNAi therapeutics are in human clinical trials. The RNAi revolution has fundamentally changed our understanding about how gene expression is regulated and has provided powerful new tools for biological research and drug discovery. It has opened the way for a new type of therapeutic with the potential to treat a wide array of diseases.

The principal challenge that remains in achieving the broadest application of RNAi therapeutics is the hurdle of delivery. The main barriers to successful RNAi delivery *in vivo* involve achieving

proper pharmacokinetics to ensure drug exposure to the relevant target cell type and harnessing mechanisms to promote cellular uptake and the release of the drug into the cytoplasm. Progress has been made on this front through the use of covalent conjugation, non-covalent complexes and nanoparticles, but targeted delivery has yet to be optimized for most cell types and tissues. Solutions will probably need to be tailored for each target cell type and disease indication. In identifying and optimizing delivery solutions, the use of reporter systems (either endogenous cell-type specific genes or transgenes) could greatly expedite the process. Likewise, in optimizing formulations, it is vital that the key parameters of efficacy and safety are assessed in parallel to rapidly arrive at the formulations which have the greatest therapeutic window.

As the challenge of siRNA delivery is met, it will be possible to advance RNAi therapeutics rapidly into clinical studies for many diseases, including some which remain untreatable or poorly treated by conventional drugs. Ongoing clinical trials of siRNA drugs for AMD and RSV infection will be the first testing ground for whether RNAi therapeutics can live up to their potential as a revolutionary new class of drugs.

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#### Competing interests statement

The authors declare **competing financial interests**: see web version for details.

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