

**R**NA interference (RNAi) is a collection of regulatory mechanisms triggered by short RNAs ranging in size from 19 to 30 nucleotides. Since the initial characterisation of RNAi in the nematode *Caenorhabditis elegans*, there has been a remarkable rate of progress in understanding the mechanisms of RNAi as well as in applying RNAi for functional genomics and therapeutics. RNAi was first shown to occur in mammalian cells in response to double-stranded small interfering RNAs (siRNAs) of ~21nt in length that serve as the effector molecules of sequence-specific gene silencing.

The key feature of RNAi lies in its ability to specifically and potently knock down the expression of genes of known sequence (figure 1 overleaf). The left side of the figure depicts the siRNA pathway in which the siRNA guide strands directs complementary base pairing to a targeted mRNA, resulting in cleavage and destruction of the message. Some small duplex RNAs are produced in the nuclei of cells and can direct targeted heterochromatin formation by directing histone modifications that lead to inactive chromatin. The right-hand side of the figure depicts the miRNA pathway in which an endogenous

hairpin precursor is processed into small duplex miRNAs, which serve as guides for directing the RISC to the 3' untranslated region of target mRNAs. The net result is inhibition of translation, but some non-targeted degradation also takes place since the RNAs are sequestered in the RNA degrading bodies called P-bodies. From the perspective of those studying gene function in mammals, this is the most powerful genetic tool available. It is now possible to silence the expression of any gene with RNAi to study the functional role of the gene product.

Although much of the success of RNAi in functional genomics has come from in vitro tissue culture studies, there are now tools for using RNA in vivo. Since RNAi triggers are small double-stranded RNAs, these can be produced by chemical synthesis or they can be produced biologically by promoter-based expression in cells of interest. 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, suggest the potential of RNAi for treating a wide range of diseases.

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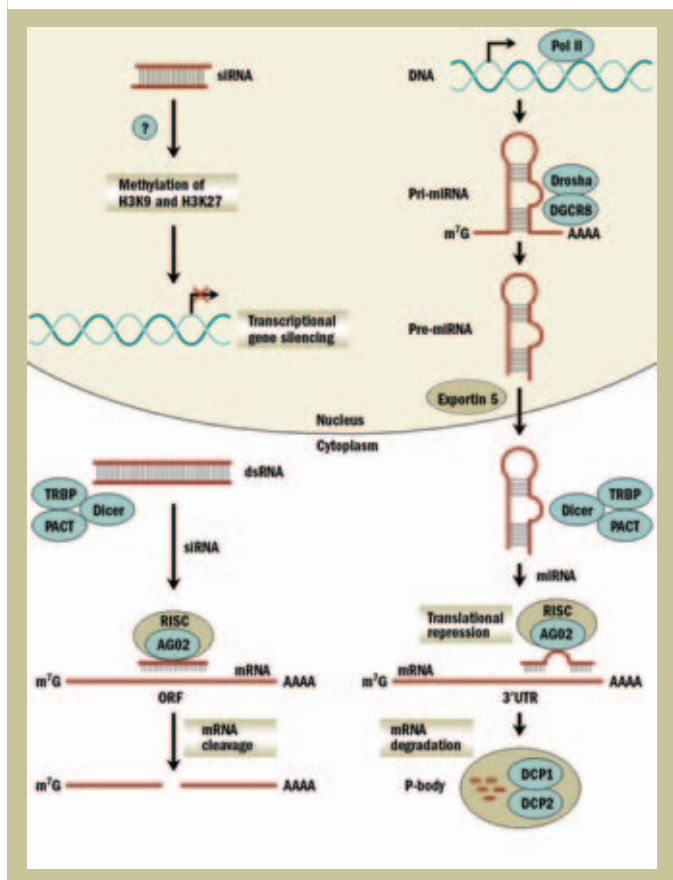
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# LOOK BEFORE YOU LEAP

**RNAi was discovered less than a decade ago and it has already proved to be a powerful genetic tool with the potential for treating a wide range of diseases. Daniel H Kim and John J Rossi discuss the potential problem areas, solutions and applications that must be overcome before it becomes a real therapeutic modality.**



Figure 1. RNAi pathways in mammalian cells.



### In therapy

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) and respiratory syncytial virus (RSV) infection. Therapies based on RNAi are also in preclinical development for other viral diseases, neurodegenerative disorders and cancers, although a number of challenges need to be addressed and improvements made for RNAi-based therapies to realise 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) 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 and saturation of endogenous RNAi pathway components, 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 RNA-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.

As well as therapeutic applications, RNAi has become an important tool in functional genomics. Libraries of small interfering RNAs or expressed shRNAs are available from a number of commercial sources, and large-scale screens with these libraries have been very rewarding. Recently an siRNA screen of human cellular targets that are required for HIV infection revealed a list of over 250 different genes that are required for HIV infection and replication in human cells. This type of information has revealed potential new targets for anti-HIV drug development, which is sorely needed. Other screens of siRNA libraries have led to the identification of key cancer pathway genes or metabolic pathway genes important in the development of several different diseases.

### Antiviral applications

The therapeutic potential of RNAi was first highlighted in poliovirus experiments demonstrating the efficacy of this approach against HIV and HBV infection in cultured cells. Soon thereafter siRNAs were shown to be efficacious in murine models for HBV replication. For HIV, in addition to targeting the virus itself, cellular genes required for HIV entry or replication have also been targeted because this avoids the problem of genetic variability in HIV that can evade RNAi by one or two nucleotide changes in the region targeted by the siRNA.

For HBV infection, delivery of siRNAs *in vivo* using lipid-based carriers has been shown to reduce viral replication in mice. For *in vivo* applications, it has been shown that extensive siRNA chemical modifications prevent interferon responses and stabilise the siRNAs in serum. In one study, the therapeutic effect of one dose of siRNA persisted for up to one week. In another study, low doses of adeno associated viral (AAV) expression vectors carrying anti-HBV shRNAs persistently inhibited HBV in mice over a period of five months, demonstrating stable and effective RNAi against viral infections.

RSV infects newborn infants and can be fatal if not treated immediately. Experiments in mice have shown RNAi to be highly effective as a method for inhibiting RSV infection. The murine studies have paved the way for human clinical trials in which the siRNAs are delivered by spraying into the lungs of healthy volunteers followed by infectious challenge of the virus. An intranasal siRNA delivery study targeting both parainfluenza virus and RSV concurrently demonstrated competition between siRNAs effective against two different viral targets. Whether competition resulted from saturation of RNA-induced silencing complex (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.

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. 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.

### Non-viral applications

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 and VEGFR1 are currently in clinical trials for the treatment of adult AMD and so far no adverse events have been reported in patients.

Spinocerebellar ataxia type 1 (SCA1) is a dominantly inherited disease that is part of a group of neurodegenerative disorders that includes Huntington's 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 vectors carrying shRNA genes targeting the SCA1 transcript. The vectors were injected into the brains of SCA1 mice leading to improvements in the pathology of neuronal cells, even under conditions that showed low transduction efficiencies.

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 has also led to long-term, stable gene silencing, along with improved survival of motor neurons and delayed onset of the disease phenotype in mice.

Hypercholesterolemia has been a proof of principle target for a couple of RNAi biotech firms. The primary target has been apolipoprotein B, part of the low density lipoprotein complex that is responsible for high levels of cholesterol in the blood. The first proof of principle for knocking out this target in vivo took advantage of the fact that linking a cholesterol moiety to an anti-ApoB siRNA allowed delivery of the siRNA into liver and jejunum, the major sites for ApoB synthesis. The injected cholesterol-siRNA conjugates both lowered the ApoB levels in serum as well as reduced the serum cholesterol levels. The disadvantage of this approach was the high concentrations of conjugates required to obtain the desired reductions. An improvement in delivery took advantage of lipid bilayer delivery vehicles, which allowed a reduction in the dose amounts. In a non-human primate model, clinically relevant reductions in serum cholesterol were achieved with a single infusion, and the reduction lasted well over a week.

Oncogenes expressed at abnormally high levels are attractive targets for RNAi-based therapies against cancers, 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. Bi-weekly delivery of siRNAs for four weeks gave an up to 50% reduction of tumour size. When RNAi therapy was combined with the chemotherapy agent paclitaxel, a reduction in tumour size of up to 90% was observed, indicating the potency and effectiveness of combining RNAi with conventional forms of therapy, especially for cancers.



Ewing's sarcoma cells have been successfully targeted in a mouse model using transferrin receptor targeted cyclodextrin nanoparticles to systemically deliver siRNAs targeting the Ews-Fli1 gene fusion. Tumour growth in vivo was suppressed after systemic delivery of siRNA-containing nanoparticles containing the transferring ligand, but not the non-targeting particles. 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.

### Business matters

Big pharma has all of a sudden become very interested in RNAi, and several significant deals between major pharma and RNAi biotech companies have transpired over the past two years. The most significant was a purchase by Merck, which paid \$1.1 billion for the acquisition of SIRNA therapeutics. Other large deals involving Novartis, Roche and the biotech company Alnylam Pharmaceuticals have also set the bar for future deals between large pharma and RNAi biotech companies.

This recent interest in RNAi by large pharma is a validation of the potential therapeutic importance of this powerful technology. There are clearly hurdles that exist, the most significant being delivery of siRNAs. The major efficacies to date have been achieved using either direct injection of siRNAs into the tissue of choice, inhalation for anti-viral applications, or the use of lipid carriers which are largely delivering the siRNAs to the liver. For tissue-specific targeting the use of RNA aptamers and antibodies have been reported in model systems. It remains to be seen whether these approaches will prove to be useful in a real therapeutic setting.

The final concern confronting commercial applications of siRNAs are intellectual property issues. There are only a handful of issued patents on RNAi, and the commercialised clinical applications of siRNAs will be severely restricted because of this. Nevertheless, the excitement of the potential of RNAi, some of which have already been realised, will continue to foster new technological developments in the field of RNAi-based drugs, and this could lead to improvements in the lives of millions. **WPF**

References available on request