

# RNAi:

## What's All the Noise About Gene Silencing?

It all began with petunias. In the late 1980s, geneticist Richard Jorgensen, then working at a California plant biotechnology company, attempted to deepen the hue of purple petunias by introducing more of the gene that gives them their color, in the form of double-stranded RNA (dsRNA). Instead, some of the engineered flowers became variegated and others turned white, indicating that expression of both the introduced pigmentation gene and its homologous endogenous gene had been knocked down or knocked out altogether. Jorgensen had serendipitously discovered an age-old natural biologic process now recognized to be evolutionarily conserved in most, if not all, forms of life. Today, gene silencing—or RNA interference (RNAi), as it is now known—has revolutionized genetics and is on the verge of spawning an entirely new class of drugs to treat human diseases with a genetic component.

The ability to selectively silence genes is one of the hottest topics in biology today. *Science* crowned RNAi as its “Breakthrough of the Year” in 2002. Nobel laureate and RNAi pioneer Phillip Sharp, who is Salvador E. Luria Professor of Biology and director of the McGovern Institute for Brain Research at the Massachusetts Institute of Technology (MIT), calls it “the most exciting discovery in the last decade,” adding that “there’s not an area of biological science this will not touch.” John Maraganore, who is president, CEO, and director of Alnylam Pharmaceuticals, touts RNAi as “presenting perhaps the broadest new class of therapeutics since recombinant proteins and monoclonal antibodies.”

Can RNAi live up to the hype? That remains to be seen, of course, but academic and industrial researchers are optimistic that it can and will, if the significant remaining barriers to its progress can be overcome. Given the rapid pace of discovery in the field, such optimism may well be justified.

### RNA Redefined

It once seemed so simple, so straightforward: basically, DNA makes messenger RNA (mRNA); mRNA makes proteins. But the discoveries associated with RNAi have shown that the real story is far more complex. RNA has been unveiled as the “man behind the curtain” in the cell, wielding previously unimagined control over and influence upon cellular processes (including gene expression and regulation) and organism development. RNAi has been revealed to be an ancient mechanism protecting cells from invading viruses and from damage by transposable genetic elements, performing a variety of cellular housekeeping functions essential to survival, health, and development.

RNAi was first described and so named by molecular biologists Andrew Fire of the Carnegie Institute of Washington and Craig Mello of the University of Massachusetts, along with their colleagues, in a landmark 19 February 1998 *Nature* paper that electrified the biology community. The team found that administering tiny amounts of dsRNA to *Caenorhabditis elegans* resulted in potent sequence-specific gene silencing. Tantalized by the possibility of acquiring a powerful new tool for genetic manipulation and analysis, investigators around the world began investigating RNAi.

The flood of significant discoveries that followed soon established the basic outlines of the mechanisms involved in RNAi. Researchers using *Drosophila* found in 2000 that long-strand dsRNA was processed in cells into 21- to 23-nucleotide snippets of RNA, which then cleaved to precisely matching homologous mRNA sequences, degrading the mRNA and effectively silencing the corresponding gene by blocking its ability to encode for proteins. The higher life forms, such as mammals, while conserving this ability, use it in different ways; the response to dsRNA is more complicated, triggering a cellular immune response involving the release of interferon that ultimately kills the cell.

Then, in 2001, Thomas Tuschl, then of the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany, discovered with his colleagues that RNAi could be prompted through the use of shorter pieces of RNA known as small interfering RNAs (siRNAs). Soon thereafter, they showed that duplexes of 21-nucleotide siRNAs mediated RNAi in cultured mammalian cells and demonstrated that siRNAs could be designed to silence specific genes without activating the interferon response. In other words, scientists could potentially silence any gene of interest in a highly predictable, reproducible, and accurate fashion.

Research scientist Gregory Hannon and his colleagues at New York's Cold Spring Harbor Laboratory contributed several key discoveries during the same period. They identified, described, and named the "Dicer" enzyme, which chops dsRNA into siRNAs, as well as the RNA-induced silencing complex (RISC), which mediates the

silencing process by degrading the homologous mRNA. In 2002, they described the use in mammalian cells of so-called short hairpin RNAs (shRNAs), which generate endogenous siRNAs within cells and thus provide stable, heritable gene silencing (in contrast, administered siRNAs are transient in their silencing effect). They whimsically named this effect "short hairpin-activated gene silencing," or SHAGging. This discovery allowed the development of cell lines and animal models with permanently silenced genes—a major step forward for basic science in general, and especially for functional genomics.

Further advances in the past few years have added the ability to silence the expression of just the mutant copy of a gene, leaving the normal copy intact, as well as to modulate the level of silencing in order to produce a range of phenotypes. Plus, researchers can now induce silencing in a controlled manner and target multiple

genes for silencing. These discoveries alone are quite important—all of these capabilities are crucial in a variety of critical applications. But some proponents believe this is only the beginning, and the best may be yet to come.

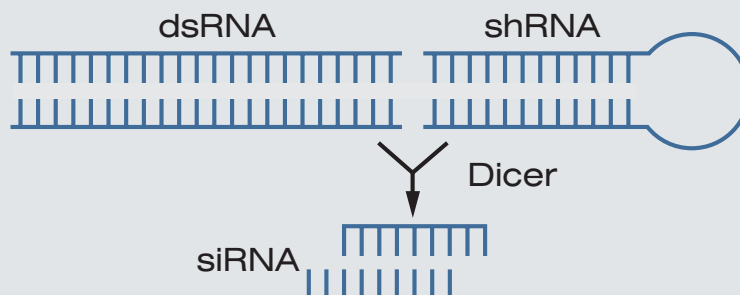
### Knock Down Genes, Drag Out Knowledge

With the continual refinement and improvement of techniques to silence genes with exquisite specificity, RNAi has already had a major impact on molecular biology. For example, the pace of discovery in functional genomics has accelerated as a consequence of researchers' enhanced ability to practice reverse genetics, in which a gene's function can be inferred by silencing its expression. With complete sequences of several genomes now on hand, including those of *C. elegans*, *Drosophila*, the mouse, and the human, investigators can now quickly, easily, reliably, and relatively inexpensively use

## Mechanism of RNAi

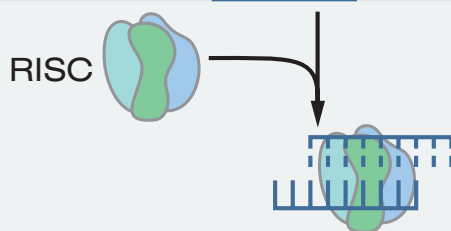
1

dsRNA and shRNA is cut by the enzyme Dicer into short RNA duplexes known as siRNAs. siRNAs are 21–23 base pairs long, with a 2-nucleotide overhang at each end.



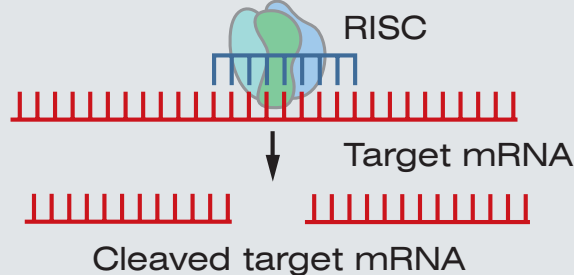
2

Inside the cells, the siRNA unwinds, and one strand is incorporated into the RISC to form a stable protein-RNA complex that can detect and destroy target mRNAs.



3

The RISC targets mRNA containing a sequence homologous to that in the siRNA. The mRNA is cleaved and degraded, and protein synthesis is interrupted.



siRNAs to silence genes of interest and determine their functions. Several companies are already selling made-to-order siRNAs for use in functional genomics work, as well as for drug target identification and validation.

The ability to knock down genes either stably (that is, creating heritable phenotypes through germline transmission of permanently silenced genes) or transiently (as opposed to knocking them out altogether) has some important advantages in the production of animal models and *in vitro* cell lines. When a gene is considered silenced by RNAi, expression is typically reduced by 70% or more. This allows the method to be used in so-called essential genes, which cannot be knocked out in animal models without killing the animal. Also, “turning down” a gene by a certain amount can sometimes more closely resemble a disease state, allowing the fashioning of more useful, refined models of some diseases.

Russell Thomas, director of the functional genomics research program at the CIIT Centers for Health Research in Research Triangle Park, North Carolina, points out another advance that RNAi has brought to animal studies: “When you knock out a gene in mice, you have to live with the consequences,” he says. “The targeted gene is knocked out for the remainder of the animal’s life span. In contrast, shRNAs with inducible promoters allow an investigator to control the timing for the knockdown of the targeted gene. With this technology, you don’t have residual developmental effects, and you can have more sophisticated experimental designs since you can look at wild-type expression and knockdown in the same animal and at multiple times throughout the animal’s life span.”

Sharp observes that RNAi will in some cases obviate the need for animal knockouts. “You can do gene knockouts in mice, and then relate phenotype to somatic cells in the human, but it’s very expensive, the gene has to be nonessential to get an interesting phenotype, and you have to ask the question in the context of the developing mouse,” he says. By comparison, he explains, RNAi allows researchers to inactivate the gene and observe in real time the changes in the metabolic, cell biologic, or other phenotype of the cell, and characterize the role of the gene in that particular situation.

Although these applications of RNAi are not yet perfected and are unlikely to

completely replace classic knockout studies, many scientists are excited about adding these new capabilities and efficiencies to their bag of laboratory tricks. “It’s funda-

[RNAi is] fundamentally changing how we do laboratory science. It will change how we do animal genetics, and we have not even scratched the surface of all the ways it will be used.

—Phillip Sharp  
Massachusetts Institute of Technology

mentally changing how we do laboratory science,” says Sharp. “It will change how we do animal genetics, and we have not even scratched the surface of all the ways it will be used.”

### RNAi and the Big Picture

The ability to reproducibly and robustly silence every single gene in the genome is expected to facilitate the acquisition of profound new knowledge regarding function and regulation at the cell and whole-organism levels. Several organizations are in the process of constructing large-scale RNAi libraries that should be available for use very soon.

Genomewide screening using RNAi libraries will help researchers learn more about global questions in systems biology, elucidating the nature and role of the complex, often interrelated pathways and signaling networks at work in organisms.

Leona Samson, director of the MIT Center for Environmental Health Sciences, has already used such a library for the 4,800 nonessential genes in *Saccharomyces cerevisiae*. She says that higher-organism RNAi construct libraries will aid toxicogenomics research by allowing researchers to interrogate each and every gene of an organism—to examine a specific gene to determine its function, or to screen large amounts of genes in the context of a specific function, to see which ones contribute to the function, and possibly what roles they might play and how they might interact. As a result, Samson says, “you can start to identify the important pathways for helping cells recover from toxic insults.”

Samson says there are important toxicogenomics treatment and prevention end points to be achieved with this type of large-scale screening. By interrogating every gene, it’s possible to compile a portrait of all the pathways that are relevant to cellular function and response, such that researchers could look at which genes are being expressed in members of a population and accurately predict the effects of exposure to particular environmental agents. “In the end,” Samson says, “we’re going to get an integrated systems view, and we need that to be able to make predictions.”

### There’s More to the Machinery

It turns out that gene silencing through degradation of mRNA by siRNAs is not the only cellular mechanism regulated by small pieces of RNA. Thanks to groundbreaking work in *C. elegans*, researchers have discovered a class of natural small RNA molecules called microRNAs that appear to be crucial in regulating development. Although they apparently use the same tools as siRNAs to carry out their functions—Dicer, RISC, and a family of proteins known as Argonautes—microRNAs differ in that their sequences do not precisely match their mRNA targets. As such, they regulate the expression of proteins by those targets, rather than degrade them altogether, which leads investigators to believe that they play an important role in the timing and nature of development—perhaps to the point of controlling differentiation in embryonic stem cells.

At Sharp’s lab, researchers discovered a set of microRNA genes that are expressed exclusively in embryonic stem cells. “As far as we can tell,” Sharp says, “they’re shut off as soon as those cells undergo differentiation. So we’re interested in what function those RNAs would have in the embryonic stem cell. And we’re also interested in devising ways to understand the specificity by which microRNAs regulate their targets.” Further, according to Sharp, it is important to elucidate exactly how a microRNA interacts with a target RNA to regulate transcription.

Tuschl, who is now group leader of the Laboratory of RNA Molecular Biology at Rockefeller University, says, “It’s a very complicated regulatory machinery that you have in your cells, with a very complicated biology behind it. We know that there are genes that express dsRNA and microRNAs, and that this gene family in humans is about 250 genes, and many of these genes are conserved. . . . The question is what all these

genes are doing, and how the RNAi machinery ties in to the gene regulation mediated by the microRNA genes.”

Recent studies further suggest there is yet a third mechanism controlled by microRNAs, an arm of the silencing machinery in the nucleus of the cell that modifies heterochromatin. The result is transcriptional repression of gene expression.

As Tuschl puts it, “This is one of the real highlights of the discovery of RNAi—that it’s a new cellular mechanism involved in regulating gene expression, and it’s as complicated and as effective as transcriptional gene regulation.” More thorough understanding of this mechanism could eventually lead to beneficial insights into development and disease processes, particularly carcinogenesis.

### Silencing Disease

Although biologists are excited about the long-range additions to knowledge that could emerge from studying the complexities of the RNAi machinery, many are focusing major efforts on exploiting what is already known—that the gene-silencing effect of RNAi holds tremendous promise in treating human disease.

RNAi therapeutics will be judged on the same criteria as any other prospective drug: potency, stability, and safety. Despite the great deal of work yet to be done, many researchers believe that RNAi-based agents will eventually pass muster on those issues, and will actually have inherent advantages over presently available classes of drugs.

For example, the fact that RNAi is a natural cellular process may mean that drugs based upon the phenomenon can be expected to be quite efficacious. “The siRNA process depends upon the endogenous pathway of microRNA biology, a process that’s present in all cells,” says Sharp. “Therefore, it’s efficient, it’s a normal biological process, and we are learning how to design siRNAs that are more efficiently taken up in that process and more efficiently used in silencing.”

Maraganore agrees. “What’s very unique about RNAi is that it’s the first natural mechanism that’s ever been discovered that would allow people to silence genes,” he says. “Because of that leveraging of that natural mechanism, you have a high degree of specificity and potency in the action of siRNAs.”

According to Nassim Usman, chief operating officer and senior vice president

of Sirna Therapeutics in Boulder, Colorado, there is ample precedent for this opinion. “If you look at the history of successful biotechnology drugs, a lot of them that have been successful are the ones that either use or replace a naturally occurring mechanism,” he says. “The two clearest examples are recombinant proteins and antibodies.”

The potential therapeutic value of RNAi has been repeatedly demonstrated in a wide variety of *in vitro* studies, and more and more *in vivo* experiments are confirming that early promise. Efficacy in gene silencing has been shown in viral diseases (such as HIV/AIDS, influenza, human papillomavirus infection, various hepatitis strains, smallpox, and SARS), neurodegenerative diseases (such as Parkinson disease, amyotrophic lateral

This is one of the real highlights of the discovery of RNAi—that it’s a new cellular mechanism involved in regulating gene expression, and it’s as complicated and as effective as transcriptional gene regulation.

—Thomas Tuschl  
Rockefeller University

sclerosis, and Alzheimer disease), cancer, inflammatory diseases (such as rheumatoid arthritis), and autoimmune diseases (such as type 1 diabetes mellitus). The intense interest in RNAi therapeutics has come at least in part because of its potential broad applicability across such a wide spectrum of disorders.

Researchers also cite the specificity of RNAi targeting: siRNAs may be able to effectively reach cellular targets that have previously been inaccessible or highly resistant to other forms of therapy. Success in reaching such targets could lead to significant advances in the treatment of several diseases with currently unmet medical needs.

### The Principle of the Thing

Researchers in academic and industrial labs around the world are pursuing therapeutic applications of RNAi. One good illustration is the work conducted by senior investigator and associate pediatrics professor Judy Lieberman and her group at the Institute for

Biomedical Research of the Harvard Medical School Center for Blood Research.

In 2003, the team published two RNAi studies of landmark importance. In one, an *in vitro* experiment presented in the *Journal of Virology* in July 2003 (issue 13), the team achieved sustained siRNA-mediated silencing of HIV-1 in primary macrophages, in essence preventing infection from taking hold. In previous studies conducted in rapidly dividing cells, such as tumors, the RNAi effect lasted only 3–5 days. In these macrophages, however, as Lieberman explains, “the silencing lasted very long, in fact for as long as we could keep the cultures growing, for some genes.”

The experiment co-targeted the viral *p24* structural gene and CCR5, the major HIV-1 co-receptor in macrophages, cells that are known to be reservoirs of HIV infection and that are stubbornly resistant to current antiviral therapies. The team found that they could completely suppress HIV replication in macrophages by using this co-targeting strategy. They also found that they could inhibit viral replication in cells that were already infected, in which HIV was integrated into the host cell. As Lieberman summarized, those are “pretty encouraging data for the possibility of using siRNA against HIV.”

The experiment also showed that RNAi holds promise as an antiviral treatment by silencing both host cell receptors and viral replication genes.

In the other major study, published in the March 2003 issue of *Nature Medicine*, Lieberman and her colleagues demonstrated that RNAi could effectively treat or prevent disease *in vivo*. They successfully prevented liver failure and fibrosis in two mouse models of autoimmune hepatitis by silencing the *Fas* gene, which encodes the Fas receptor. Many liver diseases are characterized by apoptosis, which is mediated by the Fas protein. “It turns out that the liver damage in a lot of kinds of hepatitis—even environmentally caused hepatitis, such as from alcohol or carbon tetrachloride exposure—all goes through the same final pathway,” says Lieberman. “Hepatitis B and C are not pathogenic viruses. It’s really the immune cells that infiltrate into the liver that become activated, engage the receptor that’s expressed on liver cells, and trigger death.”

The group of mice treated with the *Fas* siRNA were protected when challenged with a hepatitis-inducing agent, and in a related experiment, most of the animals in the treated group were cured after having

been subjected to a particularly aggressive model of hepatitis. “The results were pretty dramatic,” says Lieberman, “and they were obtained without any optimization.” With these and similar results reported by other investigators, it’s not surprising that hepatitis is one of the initial treatment targets being pursued by RNAi biotechnology companies.

With a \$12 million grant from the National Institute of Allergy and Infectious Diseases, Lieberman’s group, along with colleagues at MIT, is also studying the potential application of RNAi as a weapon against bioterrorism. The four projects funded by the grant include one led by Sharp to probe how RNAi and viral infection interact, another led by Lieberman designed to look at delivery questions, a third led by MIT assistant immunology professor Luk van Parijs looking at the effects of silencing various immune genes, and a fourth led by Harvard assistant pediatrics professor Prem-lata Shankar looking specifically at the possible application of RNAi against viral bioterrorism agents such as flaviviruses and poxviruses.

Successful proof-of-principle studies are a long way from proven clinical safety and efficacy, of course, but academic and industrial researchers alike are confident that RNAi therapies will move rapidly from bench to bedside. Usman says Sirna expects to file an investigational new drug application with the U.S. Food and Drug Administration for an siRNA-based drug for age-related macular degeneration later this year. That would be a remarkable milestone, considering that the RNAi phenomenon itself has only been recognized for six years.

The rapid pace of discovery and development of RNAi therapeutics should continue, according to Maraganore. “It’s a technology that, while early in terms of its discovery, lends itself to a very rapid cycle from target identification to lead optimization all the way through the start of formal investigational new drug-enabling studies,” he says.

### Delivery, Delivery, Delivery

Delivery—getting those exquisitely specific siRNAs or shRNAs to the appropriate sites in the appropriate amounts to ensure appropriate uptake and the intended silencing—remains a considerable challenge. Experts in the field agree that delivery is a daunting barrier to successful RNAi therapy. However, RNAi biotech companies and their backers are banking on overcoming the delivery barrier, and academic researchers seem confident that it can be done.

As Tuschl explains, there are basically two strategies for delivering siRNAs *in vivo*.

One strategy is gene therapy, which uses a viral vector to deliver the siRNA to the cells of interest. The other route is the chemical synthesis of the reagent, using some chemical modifications that change the properties of the siRNA such that they are more stable and are retained longer in the bloodstream; this simultaneously changes their uptake properties and allows more opportunity for uptake. With the broad applicability of RNAi to a diverse range of human diseases in a wide variety of organ systems, both delivery methods are being pursued for specific therapeutic targets.

As RNAi therapies make their way into the clinic, it is perhaps inevitable that a more traditional dichotomy will emerge in delivery: local versus systemic administration. Some organs are simply much easier than others to reach with drugs. Says Maraganore, “The delivery hurdles are going to be more significant for systemic uses of RNAi—in other words, administration of siRNAs either intravenously or subcutaneously—as compared to an approach we call direct RNAi, which is the application of siRNAs to certain anatomical sites, for example the eye or the central nervous system.” With direct RNAi, drugs can perform their actions at such sites without having to negotiate the gastrointestinal tract or other hurdles that must be faced to reach less accessible organs. Maraganore believes direct RNAi will be the first approach to yield candidates that are ultimately approved, followed shortly thereafter by approaches that use systemic administration.

The eye has been one of the first targets of siRNA therapeutics in development. Local delivery of an siRNA to the eye via intravitreal injection or topical administration is aimed at controlling the proliferation of abnormal blood vessels associated with one form of age-related macular degeneration. Several RNAi biotech companies are working on siRNAs designed to block the vascular endothelial growth factor pathway, a validated target of therapy in this disease. With the liver being another relatively easy drug delivery site, other companies are also in hot pursuit of an siRNA candidate compound to treat hepatitis C. Alnylam, meanwhile, is collaborating with the Mayo Clinic to develop an siRNA targeting the gene that encodes for  $\alpha$ -synuclein, a protein recently discovered by the Mayo Clinic and the NIH to be overexpressed in people with Parkinson disease and thought to be a causative factor in the disease. The RNAi therapy would be delivered to the central nervous system by catheterization, another local delivery option in certain situations.

In terms of systemic delivery of RNAi therapeutic agents, some researchers believe

DNA-based vectors will be the way to go. Australia’s Benitec, for example, has developed a technique it has dubbed “DNA-directed RNAi,” which it claims allows for the inducible transient or permanent silencing of multiple genes. That approach could prove beneficial in the treatment of diseases such as HIV/AIDS and cancer, in which combination therapy attacking multiple targets simultaneously is an accepted therapeutic stratagem. Given the spotty track record and regulatory scrutiny of gene therapy, however, other developers are hitching their wagons to the refinement of siRNAs themselves as drugs.

### Caution Signs

Optimism about the vast potential of RNAi must, of course, be mitigated by the appropriate scientific reserve. At least some of the delivery hurdles could yet prove to be intractable. There could be unanticipated off-target effects, in which an siRNA knocks down unintended genes, potentially provoking a toxic release of interferon.

However, experts such as Lieberman, while recognizing that “things in biology have a way of becoming more complicated than anyone can foresee,” seem confident that obstacles in RNAi’s path to the clinic can be overcome. “In the last six months,” says Lieberman, “some of the most optimistic views of RNAi—that it was completely specific, that it wouldn’t activate interferon—have turned out to not be absolutely true. But I still don’t think that they’re going to be serious problems *in vivo*.” Drug discovery and development experts point out that phenomena that might raise safety concerns *in vitro* often do not turn out to be clinically significant.

Today, RNAi is already an accepted and vital tool of the science trade, yielding important new knowledge day in and day out. In medicine, RNAi is still in its infancy, but is rising just over the horizon as one of the first tangible and widespread benefits to be derived from the sequencing of the human genome.

Aside from the potential of RNAi to benefit human health, scientists are also looking at the prospective ability of the technology to silence genes in economically important plant and animal species. For example, Japanese researchers reported in the 19 June 2003 issue of *Nature* the successful construction of transgenic coffee plants that are naturally decaffeinated by knocking down a gene involved in caffeine biosynthesis.

Now if they could just do something about those washed-out petunias . . .

**Ernie Hood**