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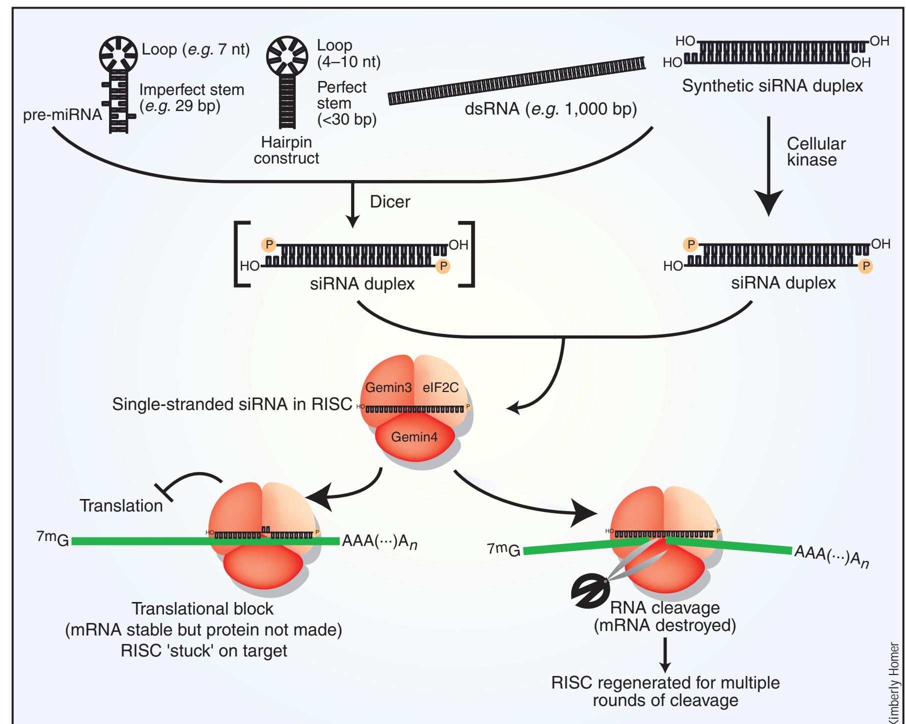
RNAi: Silencing never sounded better

The ability to trigger RNA interference in mammalian cells provides unprecedented opportunities for probing the functions of genes. Many products and resources are there to help. **Laura Bonetta** reports.

Short interfering RNAs (siRNAs) are one of the more recent additions to the stockpile of tools used to silence genes. Although their efficacy in mammalian cells was only demonstrated 3 years ago, they have quickly reached superstar status.

These molecules induce the sequence-specific silencing of genes by the process of RNA interference (RNAi) in a variety of organisms, including humans. The RNAi pathway was first discovered in *Caenorhabditis elegans*; injection of double-stranded (ds) RNA into the worm initiated sequence-specific degradation of mRNAs that contain the same sequence as the dsRNA. Later studies revealed that the cellular machinery dices up the dsRNA into shorter pieces 21–28 nucleotides in length (dubbed siRNAs) that guide a protein complex to the target mRNA. Introducing dsRNA in somatic mammalian cells induces the antiviral interferon (IFN) response, often resulting in cell death. In contrast, synthetic versions of siRNAs are effective in silencing genes.



Mechanism of RNA interference. (Reprinted by permission of Phillip D. Zamore.)

Short interfering RNAs occur naturally in cells and may serve to silence transposable elements, repetitive genes and possibly viruses. Another class of short RNA molecules found in cells, named microRNAs (miRNAs), are though to arise from precursor RNA molecules that adopt a hairpin-and-loop structure. Similar to siRNAs, miRNAs are produced by an RNA precursor that is double stranded. In mammalian cells the miRNA molecule is incorporated into a protein complex that, in most cases, inhibits translation of mRNAs that contain sequences partially complementary to the miRNA. "In animals, miRNAs usually inhibit translation, but it is not absolute," says Richard W. Carthew of Northwestern University in Evanston, Illinois. "It comes down to complementarity between the mRNA and miRNA. If they are totally complementary then the mRNA is cleaved; if not the miRNA stops translation." Indeed it appears that some siRNAs also silence genes by blocking translation and even transcription.

Once siRNAs are introduced into a mammalian cell, the amount of the corresponding mRNA typically decreases within 24–48 hours. If siRNAs are introduced in rapidly dividing cells such as HeLa cells, the targeted protein will be knocked down by 48–72 hours later. There is, however, great variability in the timing of protein disappearance, depending on the type of cell, protein half-life, efficiency of transfection and so on. In addition, different proteins differ in the extent to which they can be knocked down by siRNAs; the method can achieve 80–90% knockdown for some proteins and 50% for others.

Although this is still a relatively new technology, many researchers agree that siRNAs are more effective for silencing genes than are antisense oligonucleotides or ribozymes. "People compare RNAi to antisense, but there is a big difference," says Phillip D. Zamore of the University of Massachusetts Medical School in Worcester, Massachusetts. "This is biology. Antisense is a human invention." Because it is a biological process, "RNAi is much more predictable than antisense," says Michael Deines, vice president for marketing at Dharmacon in Lafayette, Colorado. "There seem to be rules to design siRNAs in a way that they work."

Designer siRNAs

The most popular triggers researchers use to kick off RNAi in mammalian cells are chemically or *in vitro*–synthesized siRNAs and short double-stranded hairpin-like RNAs (shRNAs) that are processed into siRNAs inside the cell.

Many companies use proprietary algorithms to design (see **Box 1** 'Help Is on the Web') and chemically synthesize 21-nucleotide-long siRNAs using a conventional DNA/RNA synthesizer. Suppliers include Ambion (Austin, Texas), ChemGenes (Ashland, Massachusetts), Dharmacon (Lafayette, Colorado), Glen Research (Sterling, Virginia), MWB Biotech (Esbersberg, Germany), Proligo (Boulder, Colorado), Qiagen (Venlo, The Netherlands) and many others. Customers often have the options of custom design or of selecting predesigned (and in some cases prevalidated) siRNAs. The most effective siRNAs can reduce target gene expression by over 90%, but some have minimal or no effect. To find an siRNA that can efficiently induce gene knockdown, most companies recommend ordering and testing three to five siRNAs per gene of interest.

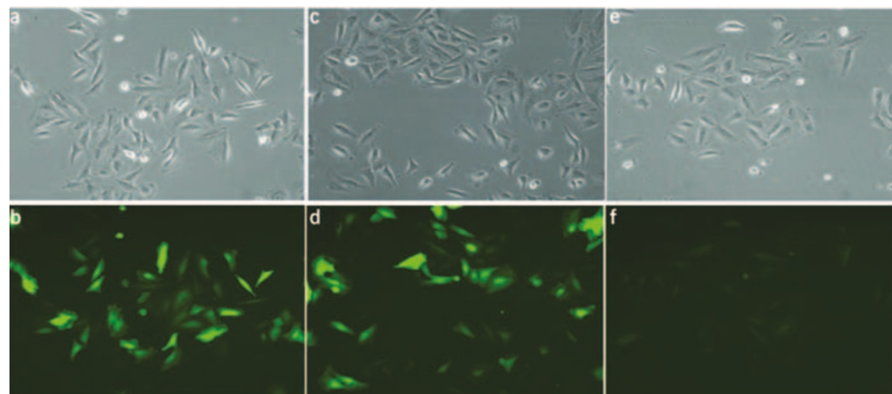
Dharmacon, the largest supplier of chemically synthesized siRNAs, guarantees that three out of four of its siRNAs targeting a specific gene will work 75% or better. In addition to individual siRNAs, Dharmacon offers *SMART*pool siRNA products, which consist of four siRNAs to the same target gene mixed together. According to Deines, the *SMART*pool products offer the "highest level of guaranteed silencing of gene and lowest chance of off-target effects." The company has pooled siRNAs for 22,000 human genes.

Companies are working to produce chemically modified siRNAs that are more effective. For example, Invitrogen has the Stealth RNAi technology. According to the company's literature, Stealth siRNAs are stable for 72 hours in serum and do not trigger the interferon response in cells.

The main advantage of ordering synthetic siRNAs is that it requires little effort or skill on the part of the researcher. In addition, chemically synthesized siRNAs are produced at a given quantity and purity, providing greater control over the amount and quality of transfected reagent in an experiment. If a researcher needs to knock down several genes, however, chemical synthesis can be an expensive proposition.

To address this problem, a number of companies, including Ambion and Beverly, Massachusetts–based New England Biolabs, provide kits for the construction of dsRNAs by *in vitro* transcription. Ambion's *Silencer* siRNA Construction Kit uses T7 RNA polymerase to generate individual strands of the siRNA. Templates for the reactions are produced from two inexpensive DNA oligonucleotides encoding the desired siRNA strands. "Price is the main reason for going this route," says David Dorris, director of RNAi technologies at Ambion.

One of the disadvantages of chemically or *in vitro*–synthesized siRNAs is that the duration of silencing depends on the rate of cell division and can be short. To get more sustained knockdown effects, researchers have the option of producing siRNA from either a plasmid or viral vector. Many groups have made siRNA adenovirus, adenovirus-associate virus, retrovirus and lentivirus vectors.



siRNA-mediated knockdown of a green fluorescent protein; panels a–d are negative controls. (Courtesy of Upstate.)

Production of siRNA from a vector is predominantly done through the transcription of a shRNA that structurally mimics an miRNA precursor. This system allows integration of the shRNA cassette into the genome to generate a stable cell lines that continuously knockdown endogenous transcripts. It also offers the flexibility to shuttle hairpin inserts from one vector to another depending on the researcher's needs.

"There are different variations of plasmid vectors in terms of promoters and selection markers, but all of them work on the same principle," says Sujay Singh, a researcher with Imgenex Corporation in San Diego, California. Imgenex sells GeneSuppressor Construction Kits that use both plasmid and viral vectors to knock down any gene. In addition, the company sells kits containing prevalidated cloned shRNA inserts in GeneSuppressor vectors together with gene-specific antibodies. Similarly, Upstate, in Charlottesville, Virginia, sells predesigned shRNA mam-

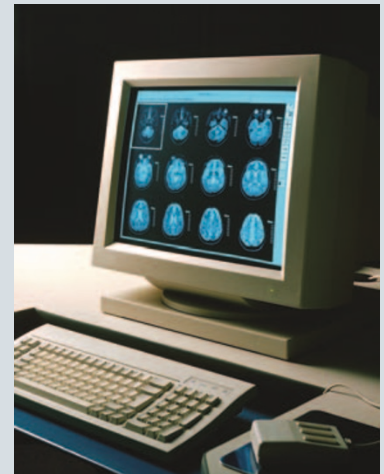
BOX 1 HELP IS ON THE WEB

The efficiency and specificity of RNAi depend on the position and sequence of the siRNA used. So how do you pick out the right 21-nucleotide sequence to knock down your favorite gene? Academic groups and companies have developed design tools, available on the Web, to help with the process.

Short interfering RNAs are duplexes composed 21-nucleotide sense antisense strands, paired in a manner to have a 2-nucleotide 3′ overhang. The sense strand does not contribute to the silencing effect (although it can contribute to off-target effects), which means that, for effective gene silencing, the antisense strand has to be incorporated into the RNA-induced silencing complex (RISC). It turns out that the relatively low thermodynamic stability in the 5′ end of the antisense siRNA strand, as compared with a higher thermodynamic stability in the 5′ end of the sense strand, leads to a bias for the incorporation of the antisense strand into the RISC. "You can favor the right strand being incorporated," says Phillip Zamore. "We are getting good at predicting how to do it."

Once the antisense siRNA strand goes into the RISC, there are some attributes of the siRNA sequence that correlate with efficiency. Dharmacon's Anastasia Khvorova identified eight such factors, such as low CG content and lack of inverted repeats[5]. More recent work by Zamore and colleagues showed that 5′ end of siRNA has largest effect on binding target of mRNA[6]. "The region more toward the 3′ end needs to be paired with mRNA for the message to be cleaved, but it is not critical for binding," says Zamore.

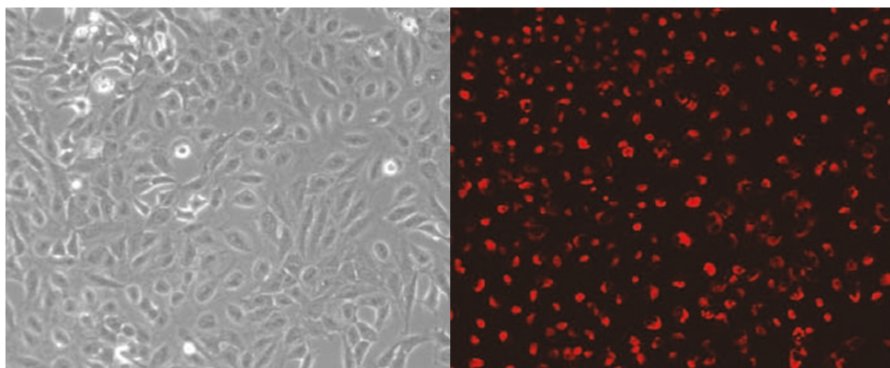
Web-based design tools incorporate these 'rules' to select 21-nucleotide sequences with the right attributes, and many will even BLAST search the NCBI database against EST libraries to ensure that the chosen sequences target only one gene. The bioinformatics group at the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts has developed the siRNA Selection Web Server (http://jura.wi.mit.edu/bioc/siRNA). Users provide the accession number of a gene or a sequence they want to target and then either select a predefined siRNA sequence pattern or opt for their own pattern. Several filters can be applied to the selection process, such as GC percentage, base variations and number of repetitive bases. "There are some defaults in place, but the user can chose his or her parameters," says bioinformatics application specialist Bingbing Yuan, who was involved in developing the site. The program produces several candidate siRNA sequences ranked by the degree of specificity. The site is available free to academic researchers, but requires user registration to limit the number of searcher per individual investigator to 15 per day. According to Yuan, updates to the site will permit the option of designing longer siRNAs and provide assistance in predicting shRNAs. Another free site for academic users is siSearch (sonnhammer.cgb.ki.se/siSearch/siSearch_1.2.htlml), offered by Eric Sonnhammer's group at the Karolinska Institute.



The RNAi OligoRetriever site (http://katahdin.cshl.org:9331/RNAi/html/rnai.html) designs DNA oligonucleotides that can be used to synthesize siRNAs by *in vitro* transcription with T7 DNA polymerase or as shRNAs transcribed from vectors containing the human U6 promoter.

Many companies that provide RNAi reagents also offer Web design tools free of charge. However, the public resources available on Dharmacon's and Ambion's websites, for example, use algorithms that are less advanced than the ones these companies use for their in-house design of siRNAs. MWG Biosciences' website offer a mechanism to convert siRNA sequences to create plasmid vectors expressing hairpin duplex RNA.

In addition, many websites offer information about how to make siRNAs and how to use them. *The siRNA User Guide*, provided by Thomas Tuschl's laboratory at The Rockefeller University (http://www.rockefeller.edu/labheads/tuschl/sirna.html), is a popular resource. Ambion's website also provides a wealth of articles and protocols for RNAi methodologies. Dharmacon will soon be publishing a book, *RNA Interference: Technical Reference & Application Guide*, that can be ordered from the company's website. "It will cover topics like the use of controls and effective siRNA design," says Michael Deines, vice president for marketing at Dharmacon. "It is meant to help researchers learn about the technique and apply it successfully."



Monitoring transfection efficiency with fluorescently labeled siRNA. (Courtesy of Upstate.)

malian plasmids for about 100 kinase genes. The company guarantees that their plasmids will silence a gene by more than 75%. Galapagos Genomics offers adenoviral shRNA reagents and guarantees a 75% silencing efficiency at the mRNA level.

Another advantage of vector systems is that they can be engineered for inducible expression. Invitrogen sells the BLOCK-iT inducible RNAi plasmid and lentivirus vectors that can be induced to express shRNA in the presence of tetracycline. Upstate is also developing a ligand-inducible plasmid expression vector, based on the synthetic ecdysone system, using technology licensed from RheoGene in Norristown, Pennsylvania.

Getting over delivery

Many researchers say that delivery is one of the biggest hurdles on the way to a successful RNAi experiment. Polyamine-based reagents or cationic lipid mixtures have been available for many years to transfect nucleic acids into cells. "But what is good for DNA and mRNA delivery is not ideal for delivering siRNA," says James E. Hagstrom, vice president of scientific operations at Mirus Corporation in Madison, Wisconsin.

One of the more popular siRNA-specific transfection reagents is Invitrogen's Lipofectamine 2000, but a slew of others are available. Mirus sells TransIT-TKO, and Upstate has recently released sIMPORTER. Novagen has amine- and lipid-based reagents in a single formulation called RiboJuice siRNA Transfection Reagent, which is meant to target a wide range of mammalian cell lines. Dharmacon will soon be releasing a whole range of transfection reagents suitable for different cells and experiments. "The efficiency of transfection may depend on cell type but also on the passage number and the confluency of the cells. The time and manner of formation of siRNA-liposome complexes are also critical," says Deines.

Transfection reagents, however, do not work *in vivo*. Viral vectors, such as adenovirus or lentivirus, carrying shRNAs inserts can be used to induce RNAi in different tissues of an animal. Mirus has developed an alternate mode of *in vivo* delivery using high-pressure tail-vein injection of siRNA in physiological solution, such as saline, to deliver siRNAs to highly vascularized mouse tissues such as the liver and muscle. The technique works with both naked siRNA and plasmid vectors. "Silencing is transient, but in some areas it lasts more than a week. You can get 30–60% reduction of gene expression in liver hepatocytes *in vivo*," says Hagstrom. Judy Lieberman's group at Harvard Medical School showed that intravenous injection of siRNAs targeting the gene *Fas* blocked the development of fulminant hepatitis in mice[1], providing the first *in vivo* evidence that infusion of siRNAs can alleviate disease in an animal model.

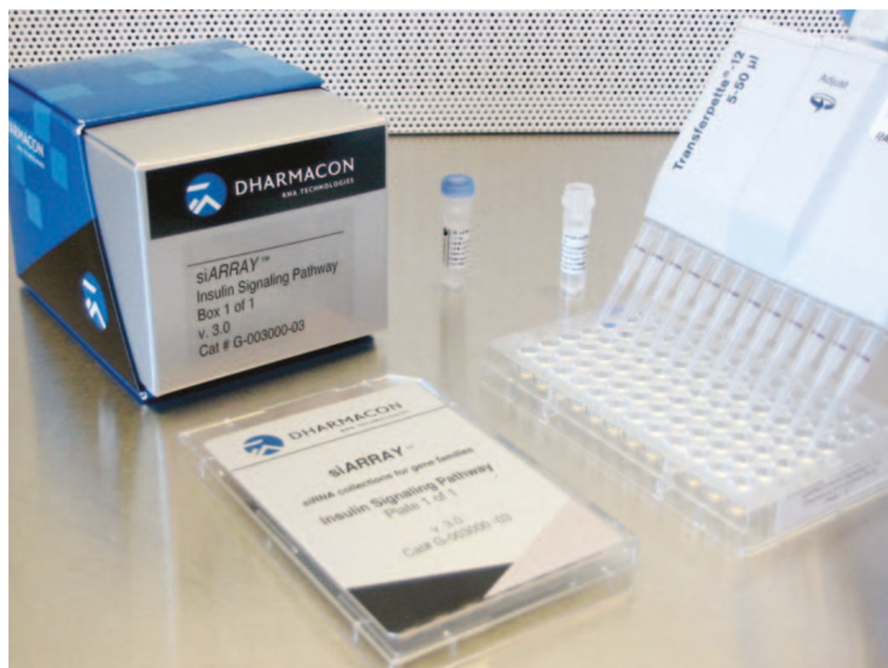
According to Hagstrom, the delivery of naked siRNAs has the advantage that no other proteins are delivered or expressed. "Nucleic acids are not immunogenic," he says. But one of the limitations of the technology is that although the liver seems to be particularly receptive to exogenous RNA, it is difficult to target other tissues. Mirus recently obtained evidence of siRNA delivery to skeletal muscle.

Mirus is now focusing on identifying siRNAs that are more stable for *in vivo* work. "We have been able to significantly extend gene knockdown times by chemically modifying siRNAs. Normally the half-life of the knockdown effect *in vivo* is 2–4 days. With chemically modified siRNA, we can extend knockdown times from days to weeks," says Hagstrom.

Controls and validation

As with any experimental protocol, having proper controls is essential. "People are typically not well informed about what controls to use for their siRNA experiments," says Deines. "We invested a lot of time and have collaborated with Agilent Technologies [in Palo Alto, California] to develop an siRNA that does not have any effects on cells as determined using microarrays." Such inert siRNAs make suitable negative controls in an RNAi experiment; fluorescently labeled versions are also available to monitor the efficiency of transfections. In addition, many companies sell prevalidated siRNAs that target housekeeping genes to be used as positive controls.

Upstate sells recombinant proteins and antibodies that can be used to validate that siRNA-mediated gene silencing worked. In partnership with Dharmacon, Upstate also offers validated *SMART*pool siRNA products as kits with corresponding antibodies. In a similar vein, Dharmacon and Genospectra, Inc. have joint forces to sell *SMART*pool siRNA reagents along with



Dharmacon siARRAY siRNA libraries. (Courtesy of Dharmacon.)

QuantiGene bDNA assays—which measure the amount of messenger RNA produced by a target gene from crude cell lysates or tissue homogenates directly, without the need for PCR.

Experts suggest monitoring both mRNA and protein levels. "Sometimes researchers will get stable knockdown of mRNA but will not see this effect because they are looking at protein levels. But for proteins with a long half-life, you may not see levels decreasing," says Imgenex director of business development Lisa Stein.

One of the worries with RNAi experiments is that siRNAs might knock down or induce genes other than the intended target. For example, an siRNAs "may be perfectly complementary to a gene but also have some complementarity to another gene and may act to silence the gene at the protein level," says Carthew. Another concern is that using high concentrations of siRNAs may trigger an interferon or other response in cells.

Helmut H.G. van Es, director of research at Galapagos Genomics in Leiden, The Netherlands, says that he has not observed off-target effects in his work but "it is important to validate results with other independent siRNAs targeting the same gene and get the same readout." The ultimate test of specificity is to insert a gene construct with a point mutation that cannot be knocked down by the siRNA and show that it rescues the phenotype induced by the siRNA." You need to build confidence biologically and molecularly," says van Es. "It is just like classical genetics," says Zamore. "RNAi has changed the way in which mutations are created. Instead of mutating the DNA, the message is cleaved, but all the same caveats remain."

Libraries of siRNAs

Until now, genome-wide screens to identify genes involved in different cellular pathways were mostly limited to nonmammalian model organisms. The advent of RNAi has made feasible to conduct similar screens in mammalian cells. Two groups—one at Cold Spring Harbor Laboratory in New York and the other at The Netherlands Cancer Institute—created human shRNA libraries that target between 8,000 and 9,000 genes and used them to identify new genes involved in two distinct signaling pathways[2,3]

Using a retrovirus-based siRNA library, the group led by René Bernards in The Netherlands identified five new genes required for p53-dependent arrest of cell proliferation. To increase the speed of RNAi screening, the researchers took advantage of the fact that each shRNA vector harbors a unique sequence identifier (bar code). The abundance of each shRNA construct in a pool of constructs could be assessed by monitoring the relative levels of each bar code using a microarray. "How long have people been looking for genes in the p53 pathway, 20 years? With one screen we get five more," says Bernards.

According to Bernards, the key to success is having a good "readout" for the screen. van Es, who is using an arrayed adenovirus-based library to target the 5,000 or so genes representing the "druggable genome," agrees. "What is important is understanding a disease so that you can

model it *in vitro* in a cellular system, preferably in human cells," says van Es. His group has used its library to identify novel drug targets for diseases such as osteoarthritis and rheumatoid arthritis.

"In the end there will be very little difference among libraries. They may differ in probe selection or vector choice, or they may target different gene sets, but the results will depend on the strength of the screens," says Bernards, whose group has conducted a dozen or so additional screens since the *Nature* publication. "In 6–10 months everyone will have all the tools to perform genome-wide screens [in human cells], but the strength of the screen rather than the strength of the library will give the advantage to one group over another."

Bernards' library is not yet available for distribution, although discussions with potential vendors are at an advanced stage. In the interim period CTR, the technology transfer arm of Cancer Research UK, has handled requests for access to the library. Hannon's library is being distributed by Open Biosystems in Huntsville, Alabama. MRC Geneservice, in the UK, recently acquired distribution rights for the United Kingdom. In addition, a number of companies have siRNA libraries targeted to hundreds of genes in specific families. Examples include the kinase libraries sold by Dharmacon and Ambion and Qiagen's druggable genome library.

The design and delivery of siRNAs have come a long way since Thomas Tuschl's group (at the time at the Max Planck Institute for Biophysical Chemistry in Gottingen, Germany) first demonstrated that siRNAs could knock down target mRNAs in mammalian cells[4]. Although RNAi-based therapies are still several years away, the time for functional genomics is here.

1. Song, E. *et al. Nat. Med.* **9**, 347–351 (2003).
2. Paddison, P.J. *et al. Nature* **428**, 427–431 (2004).
3. Berns, K. *et al. Nature* **428**, 431–437 (2004).
4. Elbashir, S.M. *et al. Nature* **411**, 494–498 (2001).
5. Reynolds, A. *et al. Nat. Biotech.* **22**, 326–330 (2004).
6. Haley, B. & Zamore, P.D. *Nat. Struct. Mol. Biol.* **11**, 599–606 (2004).

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SUPPLIERS GUIDE: COMPANIES OFFERING SIRNA DESIGN AND DELIVERY PRODUCTS

Company	Web address
Gene Therapy Systems Inc.	http://www.genetherapysystems.com/
Allele Biotechnology	http://www.allelebiotech.com/
Amaxa Biosystems	http://www.amaxa.com/rnai/
Ambion, Inc.	http://www.ambion.com/
BD Biosciences	http://www.bdbiosciences.com/
B-Bridge International, Inc.	http://www.b-bridge.com/eng/index.htm
Bioneer	http://www.bioneer.com/
Bio-Rad Laboratories	http://www.bio-rad.com
BioVision	http://www.biovision.com/
Cell Signaling Technology	http://www.cellsignal.com/
Cenix Bioscience	http://www.cenix-bioscience.com
ChemGenes Corporation	http://www.chemgenes.com/
Cyto Pulse Sciences, Inc.	http://www.cytopulse.com/
Dharmacon	http://www.dharmacon.com/
Epicentre	http://www.epicentre.com/main.asp
Eurogentec	http://www.eurogentec.be/
Galapagos Genomics	http://galapagosgenomics.com/
Glen Research	http://www.glenresearch.com/
MRC Geneservice	http://www.hgmp.mrc.ac.uk/geneservice/index.shtml
GenScript	http://www.genscript.com/
Genospectra	http://www.genospectra.com/
Genoway	http://www.genoway.com/
Imgenex Corp.	http://www.imgenex.com/
Integrated DNA Technologies	http://www.idtdna.com/
Invitrogen	http://www.invitrogen.com/
InvivoGen	http://www.invivogen.com/
Molecula	http://www.molecula.com/
Mirus	http://www.mirusbio.com/
MWG Biotech	http://www.mwg-biotech.com/
Nature Technology Corporation	http://www.natx.com/
New England Biolabs	http://www.neb.com/
Novagen (EMD Biosciences)	http://www.novagen.com/
Open Biosystems	http://www.openbiosystems.com/
Pierce Chemical	http://www.piercenet.com/
Promega	http://www.promega.com/
Proligo	http://www.proligo.com/
Qiagen	http://www1.qiagen.com/
Roche Applied Science	http://www.openbiosystems.com/
Spring Bioscience	http://www.springbio.com/
Stratagene	http://www.stratagene.com/
SuperArray	http://www.superarray.com
System Biosciences	http://www.systembio.com/
Thermo Electron Corporation	http://www.thermo.com/
Upstate	http://www.upstate.com/