THERAPEUTIC RNAi:
Delivering the Future?

When the Nobel Assembly at Karolinska Institutet awarded its 2006 Prize in Physiology or Medicine to Andrew Fire and Craig Mello for their discovery of RNA interference, it noted the process "is already being widely used in basic science as a method to study the function of genes and it may lead to novel therapies in the future." With a half-dozen or so therapies based on RNA interference currently in clinical trials, including one in phase 3 and countless more in the pipeline, the future could be closer than ever. **By Jeffrey M. Perkel**

The future for RNA interference (RNAi)—at least as it pertains to medicine—isn't here...yet. A slew of safety, efficacy and, most important, delivery issues remain to be resolved.

"As a class of strategies, this has probably gone to the clinic faster than anything in the past, but we don't have a [clinical] result to point to yet," says Greg Hannon, of the **Cold Spring Harbor Laboratory.** "No one would have predicted this funny little phenomenon would become what many view as the future of pharmaceuticals," he adds.

If the latest business news is any indication, Big Pharma certainly thinks so. **Merck & Co.** acquired RNAi firm **Sirna Therapeutics** for $1.1 billion at the close of 2006. And this past July **Roche** entered into an alliance with **Alnylam Pharmaceuticals** worth some $331 million in upfront payments and as much as $1 billion overall, and **AstraZeneca** signed a $400 million agreement with **Silence Therapeutics.**

From the RNAi companies' perspective, such deals can have profound effects, says John Maraganore, president and CEO of Alnylam Pharmaceuticals. "This is such a transformative event for our business, because it erases the need to raise capital in the public markets, and it allows us to focus our pipeline on programs we will bring to market of our own accord."

Considering the timeline, RNAi's path to the clinic has been meteoric: The gene-silencing mechanism was discovered in 1998, observed in mammalian cells in 2001, and proven to work in animals in 2003.

"I'd give it an A [grade] so far," says David Corey, professor of pharmacology at the **University of Texas Southwestern Medical Center in Dallas.** "I think things are coming along as well as might have been expected."

"Certainly not as well as naïve optimism might have suggested," he adds, "but this is the real world."

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The Real World Problem: Delivery

The success of RNAi as a research tool, and much of the excitement over its potential as a therapeutic, stem from the fact that it piggybacks on a well-characterized, intrinsic regulatory mechanism to find and degrade its cellular targets.

RNAi relies on short, double-stranded RNAs (short-interfering RNAs, or siRNAs), which are complementary to, and direct the cleavage of, specific target messenger RNAs (mRNAs) via the multiprotein RNA-induced silencing complex (RISC). Only one of the two siRNA strands, the "guide" strand, is used in this process; the other, "passenger" strand, is degraded.

"One of the things we are fortunate with in RNAi is it is a biological process, and as much as it has transformed research, we believe it will be equally transformative from a therapy standpoint," says Maraganore.

But there are some serious hurdles to overcome first, most especially delivery.

"All the challenges are in delivery," says Phillip Zamore, Gretchen Stone Cook Professor of Biomedical Sciences at the **University of Massachusetts Medical School.** Other issues, such as chemical stability, immune stimulation, and off-target effects, "are vastly overrated" by comparison, he says, not to mention irrelevant, if getting the siRNA into the target cells cannot be solved.

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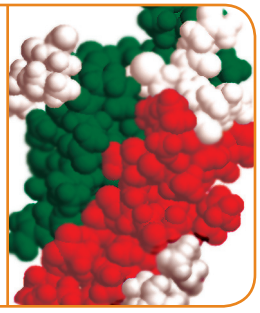

Consider off-target effects—that is, that the siRNA will target transcripts other than the intended one. Zamore notes that only siRNAs that are extensively complementary to their targets over 16 or so nucleotides can induce cleavage. Off-target effects typically arise from imperfect complementarity, which results in the less severe consequence of translational repression (as opposed to degradation).

Even if an siRNA can impact the expression of other genes, that is only medically important if those molecular changes produce a physiological effect, he says.

Besides, "I think the idea that one can create a drug that has no side effects, either molecular or phenotypic, is naïve," says Zamore.

The other common concern, stability, is also now more easily handled. "Naked siRNAs have a very short half-life," explains Judy Lieberman, professor of pediatrics at **Harvard Medical School.** First, the molecules are very small and get filtered by the kidney. And, they are highly susceptible to attack by serum nucleases.

But chemical modifications like 2´-O-methyl ribonucleotides and phosphorothioate linkages in the backbone confer resistance to nuclease attack, while enlarging the molecules to about 50 kD or so, by complexing it to other molecules or within particles, can prevent loss through kidney filtration.

"The rate-limiting problem," Lieberman says, "is how you get the small RNA into a cell." Some cells and tissues will readily take up the RNAs by endocytosis; most will not. "The bigger problem is, how do you target deep tissues or circulating cells?"

Systemic Delivery Strategies

Drug developers have hit upon several potential strategies to deal with systemic delivery. One is to package the siRNA inside liposomes, much as some transfection reagents do. Liposomes protect the siRNA from degradation and kidney clearance, but tend to get trapped in the liver, and so are mostly effective for targeting such conditions as hepatitis and hypercholesteremia.

A second strategy conjugates the siRNA to a large targeting molecule, like an antibody, aptamer, or cholesterol. Alnylam has experimented with cholesterol-linked siRNAs—which may be imported into cells via the low density lipoprotein (LDL) receptor—for systemic administration, while **Nastech Pharmaceutical** covalently couples its siRNAs to targeting peptides.

Others complex the siRNA into a particle via electrostatic interaction between the negatively charged RNA backbone and some positively charged molecule. Harvard's Lieberman, for instance, has developed a strategy in which the RNA complexes with a single-chain antibody tagged with positively charged protamine (the protein that nucleates DNA in sperm).

Using this approach, Lieberman has developed particles that target HER2-positive breast cancer and HIV-infected cells. Most recently, she targeted LFA1-positive primary lymphocytes in vitro and in mice and showed that, with the right antibodies, she can specifically target activated leukocytes. Premlata Shankar and N. Swamy Manjunath, also at Harvard, employed a variant of this strategy (using a polyarginine-tagged rabies peptide) to get siRNAs across the blood-brain barrier in mice.

Intradigm and **Calando Pharmaceuticals** use a related method. Intradigm's RNAi Nanoplex particles, for instance, comprise a core of siRNA (up to three different siRNAs may be included per particle) complexed to a positively charged carrier, such as polyethylene imine, which is then coated with polyethylene glycol and capped with a targeting molecule, says CEO Mohammad Azab. The company's antitumor therapy (currently in development) includes siRNAs to target both vascular endothelial growth factor (VEGF) and its receptor, which home in on their targets via an integrin receptor ligand.

Going Local

Most ongoing clinical trials circumvent delivery issues by focusing on local rather than systemic administration.

Leading the pack is bevasiranib, a natural (that is, unmodified) siRNA that targets VEGF mRNA. On July 12, 2007, **Opko Health** announced it was initiating a phase 3 trial to assess the efficacy of bevasiranib in treating age-related (wet) macular degeneration (AMD). The trial involves intravitrial injection—that is, into the eye.

"Bevasiranib is the most advanced drug in the field," says Sam Reich, executive vice president for ophthalmology at Opko Health. "It has been tested in a battery of toxicity studies, it is packaged as a drug. And it is the only such molecule proven to work, because it is the only one with results in any controlled clinical trial, period."

Yet it is intended for use in a very specific compartment. The eye, says Corey, "is like a test tube in the body, and a smart choice for the first foray." Injected directly into the eye, siRNAs are protected from nucleases and the immune system, and because of the compartment's relatively small volume, can be delivered at high concentration.

The first siRNA to be granted investigational new drug (IND) status by the US Food and Drug Administration (FDA), and the first to be awarded a "generic" name, bevasiranib has been tested in some 200 human patients with two different ocular indications, wet AMD and diabetic macular edema, and will find use in 330 more as a result of the new trial.

Merck's Sirna-027 (in phase 2 trials) and RTP801i-14 from **Quark Pharmaceuticals** (phase 1/2A), also intended for wet AMD, likewise are delivered directly into the eye. Sirna-027 targets the VEGF receptor, while Quark's therapy targets the hypoxia-inducible gene, RTP801.

Quark, with partner Silence Therapeutics, was recently awarded IND status for an additional siRNA, AKIi-5, which targets p53 in acute renal failure. The drug is expected to enter phase 1 clinical trials later this year. According to Alnylam's Maraganore, whose company has a business relationship with Quark, the AKIi-5 formulation could mark the first systemic RNAi approach to enter human clinical trials.

Alnylam Pharmaceutical's lead drug candidate is also delivered locally. Administered to the lung epithelia via inhalation, ALN-RSV01 (currently in phase 2 trials) is an antiviral siRNA that targets the nucleocapsid gene of respiratory syncytial virus, cause of some 125,000 pediatric and 170,000 adult hospitalizations in the United States each year.

Expression-Based Strategies

Beverly Davidson, the Roy J. Carver Biomedical Research Chair in Internal Medicine at the **University of Iowa,** Iowa City, is pursuing both siRNA- and gene therapy–mediated approaches to RNAi.

Davidson's interest is neurodegenerative diseases continued »

like Huntington's and spinocerebellar ataxia type 1. Like the lungs and eye, the central nervous system is considered a site of local delivery, and at least one company is focusing on it. **RXi Pharmaceuticals** plans to target amyotrophic lateral sclerosis (ALS, or Lou Gehrig's disease) with an siRNA against superoxide dismutase-1 delivered to the cerebrospinal fluid.

Davidson, in collaboration with Sirna, delivers siRNAs targeting the mRNAs of the huntingtin and Ataxia1 genes (mutated in Huntington's and SCA1, respectively) directly into the brain. But she also, with Sirna and **Targeted Genetics,** uses the neurotropic adeno-associated virus to deliver genes encoding those small RNAs.

In this case, the siRNA sequence is built into a larger noncoding RNA called a microRNA (miRNA), which is then expressed and processed in vivo to produce the mature siRNA.

"We are using miRNA as an siRNA shuttle, and delivering it with a virus," Davidson explains. Preliminary data in mice suggest that even partial knockdown of the targeted mRNA transcripts is beneficial, she says.

John Rossi, Lidow Family Research Chair at the **Beckman Research Institute of the City of Hope** in Duarte, California, is also working with viral delivery schemes, this time for the treatment of HIV.

Rossi's lentiviral vector encodes three small noncoding RNAs: an siRNA that targets a common exon in the HIV *tat*/*rev* gene, a ribozyme that targets the CCR5 coreceptor, and a "decoy" RNA that binds and sequesters the HIV tat protein. The virus will be used to infect (and thus immunize) hematopoietic stem cells ex vivo.

"A single siRNA can be evaded because the virus mutates so quickly," Rossi explains. "But when we combine the three approaches, it is very efficient knockdown."

Featured Participants

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Rossi says his team has initiated a five-patient phase 1 pilot study, which currently is recruiting HIV-positive individuals with AIDS lymphoma. The first patient was to be infused in early September this year, he says.

Also in clinical trials is **Nucleonics,** a Horsham, Pennsylvania–based RNAi therapeutics firm which, in May, received FDA approval to test its plasmid- (not viral-) based expression strategy. Targeting four different regions of the hepatitis B virus, the therapy is to be tested in a 15-patient phase 1 trial.

Safety First

Should any RNAi-based therapy successfully make it to the clinic, it will be a milestone achievement. But it will not be the first oligonucleotide-based drug on the market. **Isis Pharmaceuticals'** Vitravene is an antisense RNA for the treatment of cytomegalovirus retinitis. Macugen, from **Eyetech** and **Pfizer,** is an aptamer that binds to VEGF for the treatment of age-related macular degeneration.

Isis "is setting a reasonably high bar for RNAi," says University of Texas's Corey. "Double-stranded RNA must work reasonably well to compensate for the fact that it is twice as large and, thus, more expensive to make."

Indeed, RNAi drug developers have taken many of their cues from Isis, for instance with regard to chemical modifications. But Isis CEO Stan Crooke says the two classes of molecules will not necessarily behave the same. For one thing, where single-stranded molecules are amphipathic, double-standed ones are hydrophilic. Also siRNAs are larger, with possibly different biodistribution, and have a different chemical structure.

There also are safety issues to consider, says Paul Morcos, director of biology at **Gene Tools,** which develops synthetic nucleic acid analogs called morpholinos. Morcos notes that siRNAs have been shown to stimulate both the immune system's interferon cascade and the methylation of DNA and histones.

"This is kind of dangerous, because these short RNAs are recognized by cellular systems and a lot of the responses aren't good," he says.

But Zamore of the University of Massachusetts says that while some papers have raised the issue of cytokine activation by siRNAs, specific chemical modifications can apparently mitigate those responses. And Harvard's Lieberman notes that no company has yet reported any toxicity associated with siRNA delivery.

Others, meanwhile, are pushing the envelope of small RNA drugs. **Asuragen** and newly formed **Regulus Therapeutics** (a joint venture between Isis and Alnylam) are exploring drugs based on microRNAs, for instance. Neil Aronin, chief of endocrinology at the University of Massachusetts Medical School, is developing therapeutic strategies to target just the mutant copy of a gene, but not the wild-type one—a strategy that could prove useful against autosomal dominant diseases. And Isis, while retaining its focus on RNase H-based antisense technologies, is pursuing the possibility of activating RISC with single-stranded triggers, says Crooke.

Lieberman, at least, is intrigued by the idea. "I think if Isis can make something that is chemically stable and can get incorporated into RISC, it could work very well," she says.

Indeed, it may turn out to be just one of many successful strategies.

"This is a problem that will be solved many times in different ways," says Cold Spring Harbor's Hannon. "It really wouldn't be reasonable to say, 'the future is nanoparticles.' The future is probably a lot of different things."

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