RNAi Shows Cracks in Its Armor

RNAi's tendency to influence genes and proteins that it's not designed to target is provoking questions and controversy, as scientists labor to solve the problem

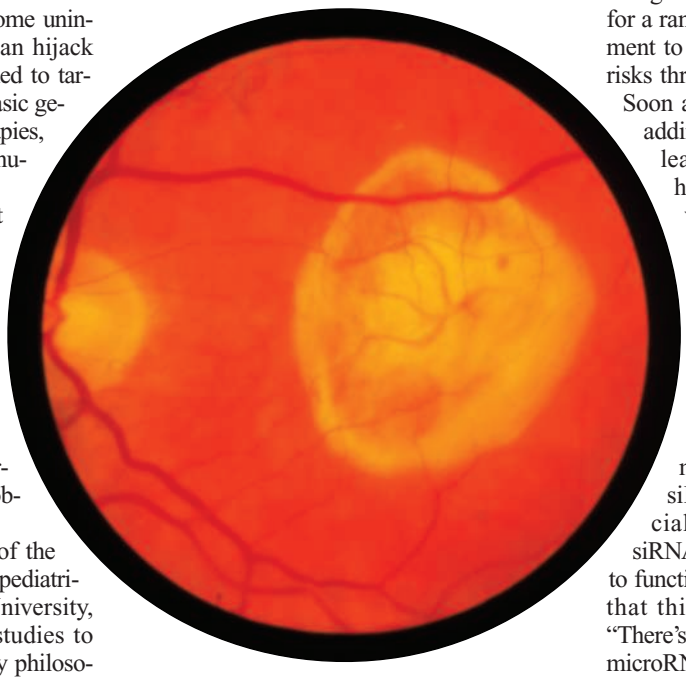
A promising new approach to manipulating genes is showing blemishes as it moves from its glamorous early days to a more nuanced adolescence. The technique, RNA interference (RNAi), shuts down genes; this braking effect helps reveal a gene's function and could potentially be used to treat a host of diseases. But a growing number of researchers are learning that RNAi, which was hailed for its laserlike specificity by scientists and the press (including *Science*, which anointed it 2002's Breakthrough of the Year), comes with some unintended baggage. In particular, it can hijack genes and proteins it wasn't designed to target—a potential problem for both basic genetics studies and RNAi-based therapies, some of which are just beginning human testing.

Even experts concerned about these so-called off-target effects hasten to point out that RNAi's future remains bright. But the issue is stirring controversy in the field. Biologists are struggling to determine—and agree upon—just how widespread off-target effects are, why they occur, and what can be done to avoid them. Some are feverishly working to circumvent the problem, with early hints of success.

"We don't know all the rules" of the RNAi machinery, says Mark Kay, a pediatrician and geneticist at Stanford University, who's conducting RNAi animal studies to treat hepatitis B and C viruses. "My philosophy is that we move forward with caution, but we move forward."

In the late 1990s, scientists discovered the potency of small RNA molecules just 21 nucleotides or so in length—some lab-produced, others naturally occurring. Injecting these RNAs, often called small interfering RNAs (siRNAs), into worms and flies silenced only messenger RNA (mRNA) molecules containing a complementary sequence. That, in turn, blunted expression of the gene producing that messenger RNA. In these organisms, there was no sign that an mRNA with a slightly mismatched sequence—with, say, 17 compatible nucleotides out of 21 in the siRNA—could also be affected.

But as scientists moved on to studies in mammals, the picture changed. One of the first to see irregularities was Peter Linsley, the executive director of cancer biology for Rosetta Inpharmatics in Seattle, Washington, a subsidiary of the drug giant Merck. "We thought it would be cool," Linsley recalls, to use siRNAs to try to design more targeted drugs. The plan: Use siRNAs to knock down expression of a particular gene that an experimental compound is already designed to target. Then add that compound to the mix, and see if it disrupts other genes as well—something that might suggest it's not targeted enough for treating patients.



Eyeing RNAi's potential. With RNAi trials launching for macular degeneration (above), researchers are watching closely to see whether the technique has any unexpected effects on humans.

But as it turned out, says Linsley, it wasn't the compounds that were poorly targeted. The siRNAs were turning down expression in multiple genes instead of just one. "The siRNAs were dirtier than our compounds," says Linsley, whose team was taken aback. The pattern persisted, and the researchers finally concluded that siRNAs could "cross-react" with other genetic targets. After some struggle convincing reviewers that the paper was accurate, it appeared in *Nature Biotechnology* in June 2003.

RNAi enthusiasts responded skeptically. After all, they'd trusted for several years that the small RNA molecules they were crafting were undeniably specific. Gradually, prodded by Linsley's work and in some cases their own, that belief shifted. "We saw more and more unexplained phenomena," says René Bernards, a cancer geneticist at the Netherlands Cancer Institute in Amsterdam. Phillip Zamore, a biochemist at the University of Massachusetts Medical School in Worcester, says his thinking evolved "when I couldn't find a way to disprove Peter Linsley." Like many of his colleagues, Zamore now believes that RNAi's limitations should have been obvious and that to presume such specificity was "incredibly unreasonable." Genetics, says Zamore, is rarely so neat.

Why off-target effects occur remains a matter of debate. One possibility is that introducing foreign siRNAs into a cell's existing RNAi system—upon which it relies for a range of functions, from early development to protecting the genome's integrity—risks throwing a wrench into the machinery. Soon after scientists began experimentally adding siRNAs to mammalian cells, they learned that these cells naturally use hundreds of so-called microRNAs, which are similarly sized and help translate RNA molecules into proteins. MicroRNAs are widely considered much less specific than siRNAs, frequently targeting sequences that only partly match their own.

This has left scientists wondering whether mammalian cells, awash in microRNAs, are mistaking foreign siRNAs for more of the same, especially because both microRNAs and siRNAs need many of the same enzymes to function. An RNAi study last year showed that this mistaken identity could occur. "There's probably a fine balance between the microRNA pathway and what we're putting into cells of animals," says John Rossi, a molecular biologist at the City of Hope Graduate School of Biological Sciences in Duarte, California.

Weak sequence matching between siRNAs and genes has also been traced to a specific part of the siRNA. That bit, called the 5′ end, helps govern how an siRNA binds to its target. As Linsley found and others such as Zamore confirmed, if that particular piece, about seven nucleotides long, matches a sequence in another gene, there's a risk of the entire siRNA binding to that gene instead.

Increasingly, biologists are turning up other seemingly esoteric details that may also determine whether an siRNA shuts down unintended genes. In the fall of 2003, a group led by Anastasia Khvorova at Dharmacon, a company in Lafayette, Colorado, and another led by Zamore, reported in *Cell* that siRNAs with certain sequences and structures unwind

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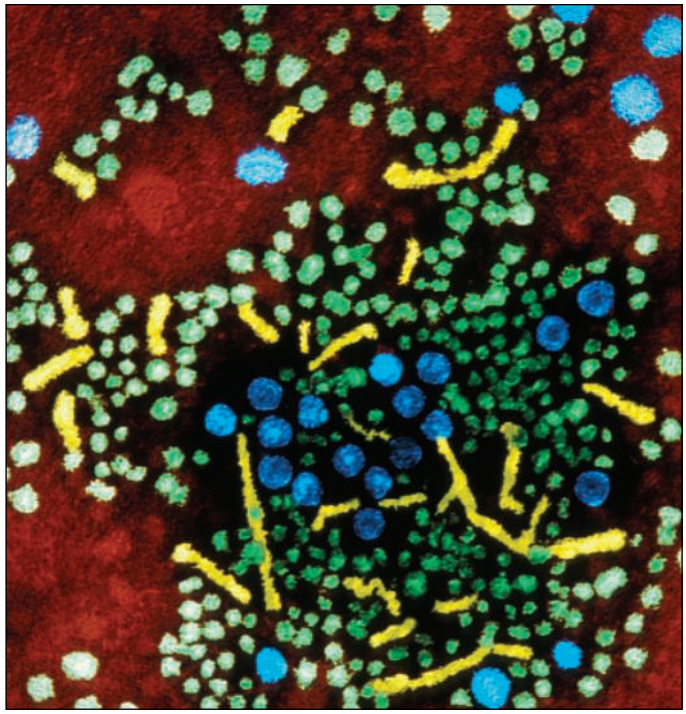
slightly differently—and the pattern in which they unwind can ultimately affect how good they are at targeting the right gene.

A year ago Bryan Williams, a cancer biologist at the Cleveland Clinic in Ohio, identified another, more controversial kind of off-target effect. In fruit fly cells and human cancer cells, he found that siRNAs activated the interferon pathway, which is the body's first line of defense against viruses.

How widespread the interferon response is remains uncertain. Although some unpublished reports of interferon response in animals exist, "by and large, people who've treated animals with siRNAs have not seen significant interferon induction," says Phillip Sharp, a biologist at the Massachusetts Institute of Technology in Cambridge and co-founder of the company Alnylam, which next year hopes to begin testing RNAi in patients with the eye disease macular degeneration.



On target? Hepatitis B is one of the diseases RNAi enthusiasts are working to disable.

In animals generally, the impact of off-target effects isn't clear. Mice with liver disease treated with RNAi technology do suffer toxic effects, says Harvard's Judy Lieberman, but those are considered more a result of the way siRNAs are delivered—in this case, under extremely high pressure, to ensure that they infiltrate liver cells. Lieberman says she's seen no visible evidence of off-target effects in her mice, but she is planning to examine the animals more carefully. Says Linsley, "You can't conclude it's not there until you look."

Looking, though, can be trickier than it sounds. For the most part, scientists are relying on microarrays, which show gene-expression levels, to learn whether their siRNAs are hitting unintended genes; in general they're finding that a dozen genes may be affected by a single siRNA. (Linsley has recorded on average at least 40.) Still, it's difficult to gauge how big a problem that is. Mismatches provoke a less dramatic change in gene expression than complete matches. Most gene expression varies by less than twofold when the siRNA doesn't fully match—often not enough to have a substantial biological impact on how a cell, or an animal, actually functions.

Cautious but upbeat. Stanford's Mark Kay hopes that off-target effects won't derail RNAi's extraordinary possibility.

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But using microarrays to look for off-target effects has one big drawback: They show only gene expression, not protein levels. If siRNAs are imitating microRNAs, that means they're not affecting DNA directly but rather are altering how RNA is translated into protein. Microarrays thus might not detect changes in protein abundance. "What's really important is what's happening at the protein level, and we don't have a lot of data on that," says Linsley.

Researchers at Dharmacon and elsewhere are trying to see whether microarray results correlate with changes in protein levels. At a meeting last week in Titisee, Germany, Sharp presented preliminary data from his lab showing a 10-fold change in protein levels with only a twofold microRNA difference, the level commonly seen from an off-target effect. But doing the kind of broad protein screens that microarrays today accomplish for genes isn't yet possible. "You can spend the rest of your life trying to see all 10,000 proteins in the cell," says Sharp, "and you'll never get an answer."

Scientists are quick to add a caveat: Even if off-target effects occur, they don't necessarily affect the phenotype, or how a cell or animal actually functions. If phenotype isn't altered, notes Zamore, the effects rarely make a difference.

The significance of off-target effects also depends on how RNAi is used: to unearth the function of mystery genes or as a medical therapy. In the first case, scientists are getting around the problem by applying several different siRNAs, each of which corresponds to a different sequence in their gene of interest. That way, if one siRNA prompts an off-target effect that changes a cell's phenotype, it will be more apparent.

When it comes to RNAi-based treatment, though, the potential challenges multiply. The first clinical trial of RNAi therapy—for use in macular degeneration—was launched last month by the Philadelphia company Acuity Pharmaceuticals. Because treatments can be restricted to the eye, the risk of off-target effects is of less concern.

For other diseases, "it's unclear how much of an issue this is going to be," says Stanford's Kay, whose RNAi work focuses on hepatitis. The disease is a popular choice for RNAi therapies because RNAi can disable the virus. Yet it's also difficult to target the liver without affecting other parts of the body. In Kay's view, RNAi therapies shouldn't be viewed differently from traditional drugs: "If you give somebody aspirin, they're going to have changes in gene expression in specific tissues." He expects that RNAi clinical trials, like all others, will need to home in on the lowest effective dose and monitor patient safety carefully.

To avoid any effects that may cause problems, researchers are chemically modifying siRNAs to try to stop them from glomming onto messenger RNAs they should ignore. Modifications can also make the key 5′ bit of siRNAs more sluggish in its binding, rendering mismatches less likely. Linsley and some Dharmacon colleagues have just submitted a paper on the subject. "We've made a few steps," he says, declining to be more specific. But "I don't think we've completely solved it." Although off-target effects may forever linger as a risk of RNAi, he and others say, they hope that they'll become less of a worry, and soon.

–JENNIFER COUZIN