

**The
Macro World
of
MicroRNA**

Short stretches of “junk DNA” are surprisingly influential in preventing or limiting disease—so influential, they are now high on the agendas of many drug companies.

by
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One reason most of us don't get cancer is that we are endowed with tumor suppressor genes. Until recently, scientists believed these genes operated through proteins that put the brakes on cell growth, thus preventing cancer's development. Now scientists realize that some tumor suppressor genes do not produce proteins at all. Instead, they make tiny snippets of RNA, called "microRNAs."

RNA, a nucleic acid, is still best known for its role as a messenger, relaying instructions from the DNA of genes to the parts of the cell that make proteins. But its many other talents keep surprising researchers. MicroRNAs, for instance—short stretches of 21 to 22 nucleotides, compared with the thousands of nucleotides that contain the recipe for making a protein—come from parts of the genome once considered worthless.

When the human genome was sequenced, only a small fraction of it—the "coding" part—seemed important. The coding DNA is first transcribed into RNA and then translated into proteins. Yet this part makes up only 1.2 percent of the 3 billion nucleotides in our genome. The rest was sometimes called "junk DNA" because it didn't seem to do anything. As David P. Bartel, an HHMI investigator at the Massachusetts Institute of Technology (MIT) points out, however, "You know it's not junk if evolution has retained it." In fact, "noncoding" DNA is beginning to look more like a gold mine—a source of many kinds of potent RNA, including forms such as microRNAs (and the different but structurally related small interfering RNAs) that help regulate gene activity.

MicroRNAs influence nearly all aspects of health and disease—the "stemness" of stem cells, cancer, early development, diabetes, viral infections, schizophrenia, heart disease, aging, and Alzheimer's disease, for example. Bartel calculates that more than half of human genes are regulated by microRNAs.

Underpinnings of Cancer

Cancer researchers in particular are excited by recently uncovered connections between microRNAs and major pathways of the disease. As many as 50 percent of all cancers involve a cellular pathway governed by the *p53* gene, a tumor suppressor. When this gene is mutated and fails to produce normal protein, malignant cells can grow wildly. In 2007, five lab groups independently reported that a family of microRNAs called *miR-34* sits in the middle of the *p53* pathway.

One team, led by HHMI investigators Gregory J. Hannon and Scott W. Lowe at Cold Spring Harbor Laboratory in New York, found that when they switched on these microRNAs in mouse cells there was a rise in cell senescence (a kind of genetic death in which cells lose the ability to replicate—see "When Cells Grow Old," page 32). Other teams showed that *miR-34* could also promote apoptosis (cell death). Both of these responses protect the organism when a particular cell's DNA is damaged through environmental exposures. By contrast, when researchers decreased the activity of *miR-34* microRNAs, cancerous cells survived and proliferated. The scientists hope that before long these microRNAs can be medically delivered to living animals in a safe and efficient manner, to disrupt cancer pathways. Their success could be a step toward preventing or treating the disease in humans.

Tyler Jacks, a cancer researcher and HHMI investigator at MIT, is also looking into the therapeutic potential of microRNAs—particularly those of the *let-7* family, which are extremely scarce in cells from mice with lung tumors. To find out whether increasing these microRNAs would reduce the development of cancer, he turned to a mouse model of lung cancer. He had already used such a model in 2005 in a landmark study of the relationship between

microRNAs and cancer. (Led by HHMI investigators Todd R. Golub at the Dana-Farber Cancer Institute in Boston and H. Robert Horvitz at MIT, the study showed that microRNAs are expressed at a much lower level in various tumor cells than in normal tissue, but it was not clear why.)

This time Jacks chose mice whose lung tumors looked like those of humans with advanced, non-small cell lung cancer. When researchers in his lab activated *let-7* microRNAs that they had delivered into the animals' lungs, "this dramatically inhibited the tumors' development," says Jacks. Unfortunately, the tumors later became resistant to the microRNAs. His group is trying to analyze why that happened.

Other researchers want to use microRNAs as early markers of cancer. Muneesh Tewari and his colleagues at the Fred Hutchinson Cancer Research Center in Seattle showed that microRNAs are present in samples of plasma and serum "in a remarkably stable form." Thus, it may be possible to monitor tumor-derived microRNAs, which are present in different amounts in healthy individuals and cancer patients, the group reported in the July 29, 2008, issue of *Proceedings of the National Academy of Sciences*.

Most promising, perhaps, are recent findings that raise the possibility of stopping a cancer's metastasis—the spread to other organs, which ultimately kills the patient. Last year, HHMI investigator Joan Massagué and colleagues at Memorial Sloan-Kettering Cancer Center identified two microRNAs (*miR-126* and *miR-335*) that were missing in the most aggressive mouse and human breast tumors. When they delivered these critical microRNAs to the breast cancer cells, the tumors lost the ability to spread. And on December 12, 2008, Arul M. Chinnaiyan, an HHMI investigator at the University of Michigan Medical School, reported in *Science* that his team had discovered that a microRNA called *miR-101* must be active to prevent the spread of prostate cancer. When this microRNA is lost, an enzyme called EZH2 that promotes the spread of cancer cells springs into action.

Thus, "for treatment purposes," Chinnaiyan says, "replacing *miR-101* in solid tumors that have lost it could reduce

their metastatic properties.” This procedure might apply not only to prostate cancer but also to breast, ovarian, and colon cancers as well as to certain forms of brain and lung cancers and leukemia. “The problem is the delivery issue,” he says. “Several drug companies are now working on ways to put specific microRNAs into ailing cells while avoiding healthy cells, where [the agents] might produce some damage.”

Many other diseases besides cancer have been linked to microRNAs. For example, at the Gladstone Institute of the University of California, San Francisco, Deepak Srivastava produced genetically engineered mice that lacked *miR-1-2*, a microRNA normally found in the animals’ heart cells. These mutants produced offspring with life-threatening holes in their hearts or fatal disruptions in their cardiac rhythms. Srivastava found that other microRNAs were deficient in mice with cardiac hypertrophy, a condition that can lead to heart failure.

In diabetes, however, scientists see the opposite problem: an overabundance of microRNAs. The goal in that case is to silence the microRNAs involved. For instance, researcher Markus Stoffel of the Swiss Federal Institute of Technology in Zurich is collaborating with an American drug company, Alnylam Pharmaceuticals, to develop what they call “antagomirs”—small fragments of RNA that can travel through the body and reduce the expression of certain microRNAs in specific organs. (HHMI investigators Phillip D. Zamore and Thomas Tuschl invented antagomirs in 2004. Zamore, Tuschl, and Bartel are among the founders of Alnylam.)

Idiosyncratic No Longer

No one realized that microRNAs would be so important when the first one was discovered, in 1993, in the microscopic worm *Caenorhabditis elegans*. Since his time as a postdoctoral fellow in Horvitz’s MIT lab, Victor Ambros had been trying to determine how the mutant gene *lin-4* made worms develop abnormally. The gene controlled developmental timing, Ambros (now on the faculty of the University of Massachusetts Medical School) and Horvitz concluded. When *lin-4*’s function was missing, this timing was off and cells

that were supposed to behave as if they belonged to older larvae got stuck at an earlier stage, repeating cell-fate programs that they should have expressed only once.

What *lin-4* normally does is turn off another gene, *lin-14*, as Ambros realized after studying the interactions between *lin-4* and *lin-14* mutations. Gary Ruvkun, also a postdoc in Horvitz’ lab, cloned *lin-14* and found that it encoded a protein which was expressed in juvenile worms. Try as they might, however, Ambros and his colleagues could not find any protein that corresponded to the *lin-4* gene.



Tyler Jacks
(top) is inhibiting lung tumors in mice by introducing the microRNA called *let-7*.

David Bartel:
(bottom) calculates that more than half of human genes are regulated by microRNAs.

Jacks: Leah Fasten Bartel: Robert E. Klein / AP, ©HHMI

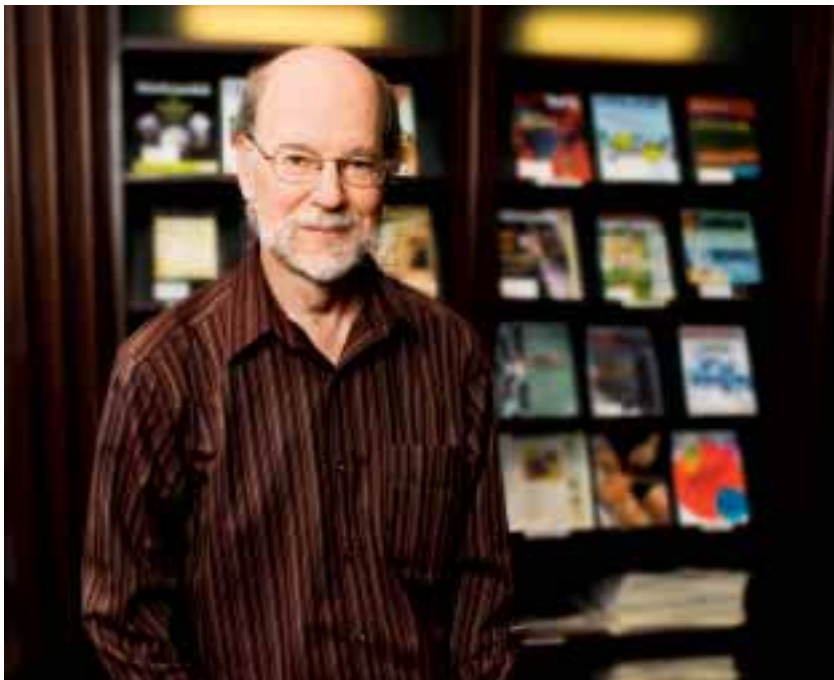


Phillip Zamore:

(top) says that knocking out microRNAs in animals in the wild will instigate very different changes than in those raised in a controlled lab environment.

Robert Horvitz:

(bottom) has shown in worms that microRNAs work in families; knocking out just one has little, if any, effect.



Then came the breakthrough in 1993, recalls Horvitz. “In his own lab, Victor made the unexpected and remarkable discovery that *lin-4* encodes a tiny, 21-nucleotide RNA. He and Gary further showed that this RNA—later recognized as the first microRNA—is complementary in sequence to its target, *lin-14*.” This observation, Horvitz says, led them to establish “a common mechanism of microRNA control of gene function—that microRNAs

act by preventing the translation of their messenger target or targets.”

“The biomedical world, however, remained indifferent,” he notes. Most biologists viewed these molecules as just “weird little idiosyncratic things, limited to developmental timing in worms,” says Joan A. Steitz, an HHMI investigator at Yale University who studies other kinds of small RNAs and is now turning her eye to microRNAs. But seven years later, Ruvkun’s lab cloned another mutant gene, *let-7*, which also encoded a 21-nucleotide RNA, and—most importantly—found that it is a

very ancient gene, conserved in a wide range of animals from flies and sea urchins to humans. (It is the same *let-7* microRNA that MIT’s Jacks now hopes to use against lung cancer.) That discovery made scientists take notice.

Shortly afterward, in 2001, a blockbuster series of three articles was published in *Science*. In these articles, three separate labs headed by Ambros, Tuschl (then at the Max Planck Institute in Göttingen, Germany, and now an HHMI investigator at the Rockefeller University), and Bartel announced the existence of “a large class of tiny noncoding RNAs” with potentially broad regulatory functions in animals. This finding started a deluge of other newly identified microRNAs.

“We keep discovering more of them,” says Bartel, who estimates that humans have at least 500 different microRNAs, which regulate thousands of genes. “Back in 2001, we were happy when we had sequenced 300 small RNAs, 55 of which were unique microRNAs. Now, with high-throughput methods, we can sequence 5 million small RNAs at a time, from humans or any animal we choose. This gives us the ability to find microRNAs that we’d missed earlier.” Those they found early on are highly conserved through evolution, according to Bartel. “As we dig deeper, we get those that are expressed at lower levels and are less likely to be conserved in other animals,” he says.

Nevertheless, in a paper published October 30, 2008, in *Nature*, Bartel and colleagues at MIT; the University of California, Berkeley; and the University of

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Queensland showed the influence of microRNAs throughout history. “MicroRNAs have been available to regulate and shape gene expression as far back as we can go in animal evolution—they might even predate animals,” he says. “They might have helped to usher in the era of multicellular animal life.”

Exploring Biological Mechanisms

To find out what microRNAs do biologically, scientists have been knocking out the known microRNA genes in worms. In December 2007, Horvitz, Ambros, Bartel, and their colleagues reported in *PLoS Genetics* on a new collection of engineered worms in which 95 of the 115 microRNAs that had been clearly identified in *C. elegans* were knocked out.

“To our surprise, the worms in general seemed perfectly okay,” Horvitz says. The researchers looked for worms with abnormal behavior or anatomy but, apart from those with deletions in *lin-4*, *let-7*, or *lxy-6* (identified earlier by Oliver Hobert, an HHMI investigator at Columbia University), they could find only one—a worm that was constipated. This worm’s “abnormally long defecation cycle” was caused by the deletion of two microRNA genes, *miR-240* and *miR-786*, both of which are conserved in other species.

“The microRNAs are in families,” Horvitz explains. “If you knock out the entire family, defects can appear,” but knock out just one microRNA and in most cases the worms look normal. To see a change, he says, it takes not just single or even double knockouts, but at least triple, and perhaps six or seven knockouts in a single animal. “We can do this in the worm,

but imagine how difficult it would be to do it in a mouse!”

It takes much longer—about a year, as opposed to a week—and costs a great deal more to produce a mouse with a specific gene deletion. Nevertheless, the Sanger Institute in Cambridge, England, is creating a library of knockouts of each of the 500 microRNAs identified in the mouse genome so far. This resource will serve as a counterpart to the National Institutes of Health Knockout Mouse Project for protein-encoding genes. Eventually, stem cell lines of each mouse in each library will be available to scientists who want to use these rodents as models for human diseases.

Meanwhile two studies—one led by Matthias Selbach and Nikolaus Rajewsky of the Max Delbrück Center for Molecular Medicine in Berlin, Germany, and the other by Bartel and Steven Gygi of Harvard Medical School—have measured how proteins change after a cell encounters a specific microRNA. Using a new technique called SILAC (an updated version of mass spectrometry), they examined several thousand proteins and concluded, in the September 4, 2008, issue of *Nature*, that a single microRNA can repress the production of hundreds of proteins—but for each protein this repression is relatively mild.

Yet even small changes in protein expression can make a huge difference, says Phillip Zamore, an HHMI investigator at the University of Massachusetts Medical School. Zamore is comparing highly selective microRNAs with those that control

many genes at once. He points out that animals in which researchers have knocked out a gene—such as the fruit flies he studies in his own lab—are well fed and live in temperature-controlled environments. But what if a fly that is less than perfect is put in a normal environment? “In real life, flies experience noonday sun and cold nights. And their proteins are distributed in gradients that change with temperature,” he says. “Something may look like a small defect in the lab, but you know what would happen in the wild? The fly would die.”

Everything depends, of course, on the microRNAs’ targets. MicroRNAs cannot act until they have paired with complementary nucleotides on a cell’s messenger RNA. Once paired, they generally shorten the lifetime of the messenger RNA, or block or weaken its instructions, thus reducing the output of protein. Now, one big push in microRNA research is to find an efficient method of zeroing in on their targets.

Several groups have developed computer programs that predict which genes are the most likely targets of each microRNA. To evaluate these programs and find targets that might be missed by the programs, researchers are also developing new methods for identifying targets.

At Stanford University, for instance, HHMI investigator Patrick O. Brown is using the kind of microarray chips he pioneered 14 years ago to examine large quantities of messenger RNA, looking for targets. Brown’s results show that a program developed by Bartel and colleagues does a relatively good job of predicting the targets of the microRNAs he examined. He suggests that many segments of messenger RNA are ready to be “tuned” by specific microRNAs, as needed, in a “continuous scale of regulation.”

And former HHMI investigator Jennifer A. Doudna (now at Genentech) is attacking the problem from a different angle. Her UC Berkeley lab is looking at what allows a specific microRNA to target a specific messenger RNA in terms of both nucleotide sequence and structure. “We want to harness microRNAs and manipulate them for use in therapy,” she says. “Potentially, it could have a large impact on the way drug companies develop microRNAs.” ■