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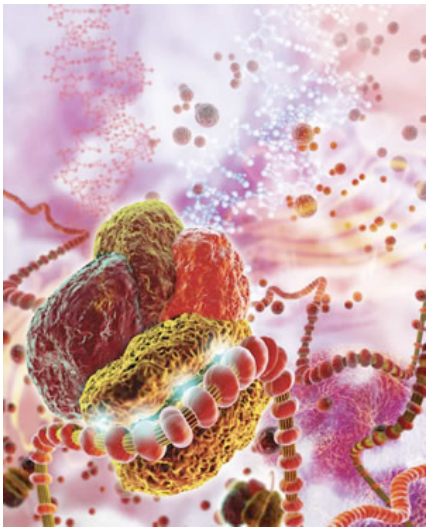
By Judy Lieberman

Master of the Cell

RNA interference, with its powerful promise of therapy for many diseases, may also act as a master regulator of most—if not all—cellular processes.



RNA silencing. Computer artwork showing a length of RNA (yellow with red rings) bound to an RNA-induced silencing complex (RISC).
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One of the biggest surprises in biology in the past decades was the discovery that humans have about the same number of protein coding genes as a worm. That puzzling finding began to make sense when we realized that we were missing a big part of the picture: a lot of DNA is transcribed into RNA but never into proteins. The more we learn about these RNAs, the more we realize how much complexity they add. Some of these noncoding RNAs, called microRNAs because of their small size, interfere with protein expression by chopping up protein coding transcripts or inhibiting their translation into proteins. Their effect on cell fate and function is far wider than we initially thought. In recent years, it has become clear that microRNAs can act as master switches by regulating large networks of genes.

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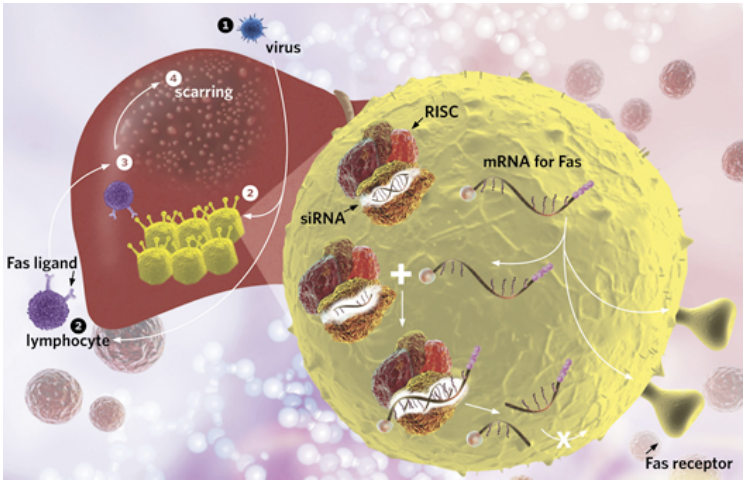
I came to work on microRNAs by a circuitous path. I started as a theoretical high-energy particle physicist, but after 8 years decided to go to medical school to do work that more directly helped people. As part of my medical training in hematology and oncology, I began a postdoc at MIT in the lab of Herman Eisen in the early eighties when molecular biology was just coming into its own: The T-cell receptor had just been discovered (work to which the Eisen lab contributed), and HIV was about to be identified as the cause of AIDS. With no therapy available then, AIDS patients died a truly gruesome death. The Eisen lab studied the cytotoxic T cells that were supposed to protect us against viral infections like HIV.

After my postdoc, I was offered a job at Tufts–New England Medical Center that combined clinical work in hematology with running a lab. I decided that my new lab would work on understanding the T-cell response to HIV and why these cells fail to control the infection, with an eye towards

developing immune-based therapy. We also investigated how cytotoxic T cells activate programmed cell death (apoptosis) in virally infected cells.

I was immersed in HIV and T-cell immunology work in 1998 when I read the Fire and Mello paper[1] describing one of the first examples of RNA interference (RNAi) in *C. elegans*. I was intrigued and perplexed by the paper: how could a double-stranded RNA possibly silence gene expression? I would periodically ask a colleague working on worms, Keith Blackwell, if there was an explanation for this strange phenomenon.



Liver saver

Liver damage, by infection with hepatitis B or C virus (1) or other causes, doesn't usually result from the first insult. In the case of infection, for example, the virus initiates an inflammatory response, which upregulates Fas receptors on liver cells (2), and attracts T lymphocytes. T cells express the ligand for Fas on their surface and when they enter the liver they bind the Fas receptors (3), initiating the apoptosis pathway that leads to tissue scarring (4). When the small interfering RNAs (siRNA) were taken up by liver cells, they degraded the RNA message for the Fas receptor, preventing even the most deadly and acute liver damage.

My curiosity was thus piqued when 3 years later Carl Novina, a postdoc in Phillip Sharp's lab whom I had known when he was a graduate student at Tufts, came to me with the still-unpublished news that RNA silencing also functions in mammalian cells. Tom Tuschl had found that genes could be silenced by introducing small double-stranded RNAs into a cell. Carl wanted to find out if this method could be used to inhibit HIV infection of immune cells. The prospect was tantalizing. What if we could use small interfering RNA to block HIV infection in humans?

Because T cells—the natural target of HIV in the body—are difficult to transfect, we started by trying to block infection in an epithelial cell line that was engineered to express CD4 and CCR5, two receptors required for HIV infection. Transfecting the cells with a small interfering RNA (siRNA) designed to degrade the messenger RNA (mRNA) for CD4 indeed blocked HIV infection by 4- to 10-fold. Encouraged by these results, we used RNA silencing to target an HIV gene that encodes for the viral capsid, and found that we could knock down both the host mRNA and the viral mRNA within the host cell. Knocking down either gene could stop the spread of the infection in cell culture. It was no small achievement. Our paper was one of the first in the field to show the potential of RNAi in treating human disease.[2] Of course, at the time, no one understood that RNAi is actually a very basic antiviral mechanism. Organisms, like plants and more primitive animals that don't have adaptive immune systems, use RNAi to attack and degrade viral mRNAs. In retrospect, it made a lot of sense that our HIV experiment would work.

RNA interference is much more than just a cell's antiviral technique. This mechanism acts as a master regulator of gene expression, directing a cell's response to developmental and environmental cues.

I was excited about translating our promising in vitro results into therapies, but in 2001 there were still no good small animal models of HIV infection. Without my knowledge, my postdocs, led by Erwei Song, decided to test the concept of using RNAi to protect against a different disease in mice—hepatitis.[3] Two groups had shown that rapid intravenous injection of siRNAs in a large volume (so-called "hydrodynamic injection") in mice was able to knock down expression of a simultaneously injected luciferase transgene in some organs in the mouse. The most effective knockdown was in liver cells. Using RNAi to prevent hepatitis might work. Erwei and his friends in China, who were skilled at performing the exceedingly tricky hydrodynamic injections, tried to silence a transcript for one of the

caspases. This enzyme triggers programmed cell death in liver cells in virtually all forms of hepatitis. However, it didn't work and all the mice died. When Erwei finally told me what had happened, I thought the approach was promising, but suggested trying to knock down a different target.

The RNA interference mechanism acts as a master regulator of gene expression, directing a cell's response to developmental and environmental cues.

Because of my research on apoptosis in the immune system and antiviral immunity, I knew that liver cell death in hepatitis, no matter what the cause, is triggered by activating a death receptor called Fas on liver cells. Infection with hepatitis B and C viruses, for example, does not kill liver cells directly. Rather, the inflammation they cause induces Fas expression on liver cells and attracts killer lymphocytes bearing the counter-receptor for Fas (called Fas ligand) to infiltrate the liver, where they attack Fas-bearing liver cells (see graphic above). Triggering Fas is the common pathway for liver damage. Therefore knocking down Fas at the beginning of the pathway seemed like a good idea.

After hydrodynamic injection of siRNAs to knock down expression of the Fas receptor, Fas expression was reduced by 80% throughout the liver. As a consequence, there was a dramatic reduction in liver-cell damage. The technique could prevent liver damage not only in models of chronic hepatitis, but also in an acute liver damage model, in which all mice normally die within 3 days.[4] After knocking down *Fas* in the liver, most mice survived the lethal challenge and recovered. It was clear that knocking down the Fas receptor could potentially block damage from any kind of hepatitis insult. What was more impressive was how easy it was to get these experiments to work. Once we started looking at Fas, we finished all of the experiments in the study in a month or two. When things work that well, it gives you the sense that you're looking at a really fundamental process, rather than a curious side pathway. I was very excited and optimistic that small RNAs could be the basis for a new type of drug.

Research soon emerged showing that developing RNAi drugs wouldn't be quite so easy. The active small RNAs, called small interfering RNAs or siRNAs, mediate RNAi silence genes by binding to a matching messenger RNA (mRNA) sequence and cutting it. But researchers found that a single siRNA could silence other mRNAs—not just the ones being targeted. These off-target effects could arise from one of two mechanisms: siRNAs were either hitting unintended genes that share partial sequence complementarity, or they were triggering the intracellular immune sensors that recognize viral double-stranded RNAs, causing inflammation and widespread immune stimulation. These potential problems were rapidly addressed by others who found that chemical modifications of the siRNA sugar backbone could block most off-target effects without jeopardizing gene knockdown. The other obstacle, which is still a major problem, was the incredible difficulty getting cells to take up naked RNAs. Hepatocytes were relatively easy because the liver is the filtering organ of the body, with a rich blood supply that routinely takes up particulates.

We tried to address some of these issues while working on an RNAi-based microbicide to prevent sexual transmission of viral infection (and ultimately HIV) in mice. Because of the lack of a small animal model for HIV transmission at the time, we decided to first try to block herpes transmission in mice. We developed a way of getting siRNAs into epithelial cells by either mixing them with a transfection lipid used to introduce exogenous nucleic acids into cells in the lab or by adding a cholesterol tag to the end of the RNA sequence that allowed the RNA to be taken up into cells. The result: effective gene silencing of an epithelial cell receptor that the herpes virus uses to enter the cell. The method could actually protect mice from a lethal vaginal dose of HSV-2 without causing immune recognition of the siRNA.[5] However, neither of these methods was effective at transducing the T cells that HIV infects; we are still testing ways to modify siRNAs that could prevent HIV transmission, with some promising leads.

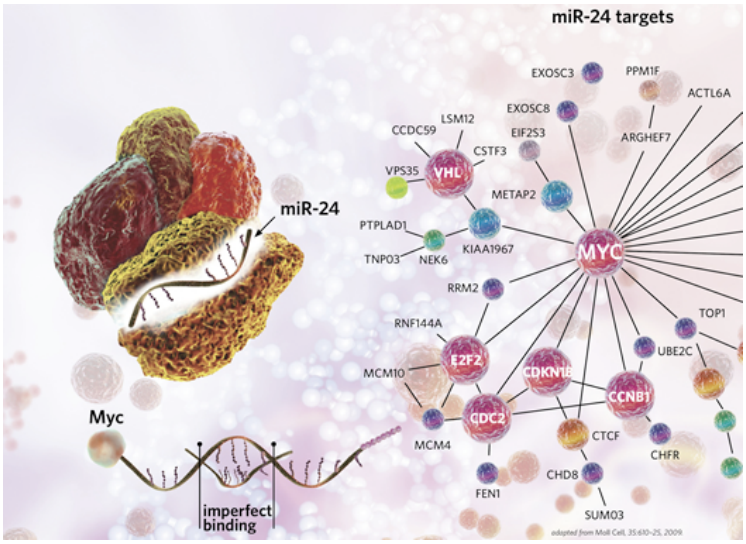
Erwei Song finished his postdoc in my lab in 2004, and returned to China to work as a breast cancer surgeon. When I visited China in 2005 for the annual meeting of a US–Sino comprehensive HIV research program in Beijing that I had helped organize, Song came to see me with exciting news. He had been working at Sun Yat Sen University in Guangzhou on his own projects and told me that he had discovered a method for culturing cancer stem cells from breast tumors. At the time, the cancer stem-cell hypothesis, which posits that breast cancer is initiated by a rare population of cancer stem cells, was controversial (and still is), in part because these cells are hard to identify and because the mouse models for human cancer might not accurately reflect how cancers originate in a human. These cells are relatively resistant to current chemotherapy and radiation therapy. They survive after cancer

therapy and replenish the cancerous mass, leading to relapse. Although some tumors may be formed by initiating cells that do not resemble stem cells, it is likely that stem cell–like tumor cells are more aggressive at forming tumors, with a higher likelihood of relapse and metastasis.

With his success at culturing these elusive cells, I couldn't help but wonder what their microRNA expression profile looked like in comparison to other cells. microRNAs, the small endogenous RNAs that mediate RNAi naturally, were first identified in seminal studies by Victor Ambros and Gary Ruvkun in 1993, 5 years before the Fire and Mello paper. Their work suggested that small RNAs are instrumental in regulating development, as would later be confirmed by studies in several model organisms. I had a hunch based on this work that microRNA expression would be different in breast cancer stem cells than in more differentiated tumor cells or normal tissue and that it would change as the stem cells differentiated to form a tumor.

> **It appeared that we had found not only a potential therapeutic mediator, but also a factor that controlled cancer cell "stemness".**

Our collaborative effort revealed that the breast cancer stem cells expressed far fewer microRNAs than their more differentiated counterparts. One family of microRNAs stood out: the let-7 family containing 11 related sequences in humans. let-7 is one of the most evolutionarily ancient microRNAs that Ruvkun's lab had shown regulates the larval-to-adult transition in worms. The more we looked at this microRNA in functional assays, the more interesting it became. let-7 was not expressed in cancer stem cells, but its expression increased as the cell differentiated. When we infected cancer stem cells with a lentivirus expressing let-7, we could force their differentiation into treatment-susceptible cancer cells. Surprisingly, forced expression of let-7 also reduced the number of tumors formed in the mouse and reduced their metastases.



Master of the cell

Current computer algorithms used to search the genome for mRNA targets of microRNAs rely on the idea of a perfect sequence match between the mRNA and the seed region (nucleotides 2—8) of the microRNA. In reality, some targets are "seedless" and may have good pairing elsewhere in the sequence. By looking at the mRNA sequences that were downregulated when miR-24 was expressed, we found not only that this miRNA targeted major transcription factors in the cell cycle pathway like MYC and E2F2, but it also regulated genes that were transcriptionally regulated by MYC and E2F2.

It appeared that we had found not only a potential therapeutic mediator, but also a factor that controlled cancer cell "stemness." In fact, let-7 controlled a number of stem cell properties, including the ability to self-renew and differentiate into different cell types (or "multipotency"). It accomplished this task by regulating the expression of more than one gene. Frank Slack had previously identified the oncogene RAS as a target of let-7 and David Bartel's group had identified another oncogene, HMGA2, as a target. We found that let-7 regulation of RAS contributed to loss of self-renewal, while knockdown of HMGA2 led to loss of multipotency. Our study suggested that let-7 might be a master regulator of defining cancer stem cell properties.[6] Together with the earlier studies in worms, this suggested that let-7 might be a master regulator of "stemness" more generally. At the time, researchers regarded small RNAs as rheostats that fine tune gene expression and cellular function; they were thought to only make small adjustments in expression. When I wrote the let-7 paper and called it a "master regulator," controlling the very identity of a stem cell, I was asked to change the wording. However, I was convinced that these small RNAs are more powerful than the field had acknowledged.

While we were examining microRNAs in breast cancer stem cells, we were also looking at their role in blood cell differentiation from immature progenitor cells—somewhat more familiar territory for me. We became especially interested in one microRNA—miR-24—that stood out because it is upregulated as multipotent blood cells differentiate into a wide variety of mature blood cells. These mature cells are no longer capable of proliferating. Introducing miR-24 into proliferating normal and tumor cells also stopped them from further cell division. To understand how miR-24 worked, we wanted to identify the genes it regulated. It was a challenge, as microRNAs are only ~22 nucleotides long and bind to their targets by matching their sequence to a sequence in the target mRNA. But it's a loose match at best—not every base pair matches its complementary nucleic acid. The algorithms used to predict which mRNA targets will be regulated by a particular microRNA are based on sequence matching. This often identifies thousands of potential targets and sometimes misses important ones like the oncogene RAS as a target of let-7. The algorithms place a lot of emphasis on target mRNAs that contain an exact 7 or 8 nucleotide match in their 3´ untranslated region (UTR) to residues 2–8 of the miRNA, called the "seed" region. Instead, we looked at all mRNAs that were downregulated when miR-24 was expressed in cells that don't normally express it. We found 248 downregulated mRNAs, which did not overlap much with those found by the prediction algorithms.

> **I am optimistic that RNAi will be harnessed to produce a new class of drugs to treat many diseases.**

To make sense of the large list of potential targets and choose a small number of genes to test experimentally, we collaborated with Winston Hide, a bioinformatician at the Harvard School of Public Health. Not only did miR-24 suppress major transcription factors that regulate the cell cycle—E2F2 and MYC—it micromanaged the expression of many of the transcripts that E2F2 and MYC activated (see graphic above). Most of the downstream genes we looked at experimentally were regulated by miR-24 recognition of "seedless" complementary sequences.[7] We now think that miR-24 is another example of a "master regulator" of the cell, which acts by directly suppressing the expression of many genes that act in interconnected pathways.

Introducing microRNAs, such as let-7 or miR-24, that force cancer stem cells to differentiate or cause cells to stop dividing could be used for cancer therapy. Let-7 could make tumors more susceptible to standard cancer chemotherapy or radiation. Targeting cancer stem cells, especially, might address this highly malignant and refractory source of recurrent tumors. This is an approach we are now working on.

Jumping into RNAi research as it was just beginning has been extraordinarily rewarding. As I move into new fields, however, I've never given up on trying to understand the questions that I asked when I started my lab. Although I completely abandoned theoretical particle physics, I am still deeply involved in understanding how HIV manipulates and overcomes antiviral immunity, and how antiviral killer lymphocytes destroy their targets. As a physician, I hope that solving these questions and understanding how microRNAs work can be used to improve treatments for HIV and cancer. In my work on RNAi, and indeed during the 25 years I have been engaged in biomedical research, I have had one foot in basic research and the other in translational work, seeking to apply new understanding of biology to improving patient treatment. I am optimistic that RNAi will be harnessed to produce a new class of drugs to treat many diseases.

Judy Lieberman is a senior investigator at the Immune Disease Institute and Program in Cellular and Molecular Medicine, Children's Hospital Boston and a professor of pediatrics at the Harvard Medical School.

Have a comment? E-mail us at mail@the-scientist.com

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D. Hollenberg's article is available online
by Jason Halm

[Comment posted 2010-04-28 13:51:23]
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D. Hollenberg
On the Evolution and Dynamics of Biological Networks
6,00 ? aggiungi al carrello

Dennis Hollenberg

On the Evolution and Dynamics of Biological Networks

Abstract. Large numbers of interacting non-genic molecules regulate metabolism and embryonic morphogenesis through often unspecific mechanisms. This lack of specificity suggests that the prevailing viewpoint, that such ordered processes result from the direct control of genes and their products irrespective of local molecular dynamics, is incomplete. Proposed here is a hypothetical type of control dynamics, called indirect, that is exhibited in natural biological networks of interacting and adapting elements. Evidence in the literature suggests that ordinary interactions among such elements ? including organisms, cells and molecules ? produce six network phenomena that can be attributed to indirect-control dynamics. Although these hypotheses can be disproved, including by showing that the phenomena can be accounted for by an alternative process, the set of ecological dynamics argued to underlie the phenomena is observable, biologically consistent and universal. In contrast, the direct-control dynamics required by the modern synthesis likely is biologically disadvantageous. This biological view of networks suggests new areas of research.

LINK

To Dr Hollenberg
by anonymous poster

[Comment posted 2010-04-07 15:44:54]

Dr Hollenberg,

Some months ago I tried to get your article to read it and had difficulties to obtain a pdf. Would you please send me a pdf ?. I would be most appreciative. Enclosed please find my e-mail:

soydealmodovar@hotmail.com.

Thank you

RNAi revolution
by Hanoch Slor

[Comment posted 2010-04-06 09:59:12]
Dr Judy Liberman deserve a medal of excellence, if there is one, for her inquisitive mind and approach to study this intriguing system.
I shall be using this paper in my Human Molecular Genetics class as well as in a class of advanced seminar in biotechnology.
All the best Judy (Kol Hakavod).

Prof.(emeritus) Hanoch Slor, Israel

Jack of all, Master of none
by anonymous poster

[Comment posted 2010-04-06 09:23:49]
Hollenberg's comments smack of a "God Complex"- and the verbal acrobatics displayed makes it less understandable to inferior species of scientists that include me.If the secret of what is the master regulator has been revealed in the 1977 paper (Is this the one banned in the US?), Dr.Prof. Hollenberg, start preparing your acceptance speach at the Nobel.

Evolution Drives All Cosmic Faring Programs
by Dov Henis

[Comment posted 2010-04-03 10:01:36]
Evolution Drives All Cosmic Faring Programs

A. "Master of the Cell"
LINK

B. "On RNA Cell Faring Programs"
LINK

C. "03.2010 Updated Life Manifest"
LINK

and

"Genomes Are RNAs-Made Patterns-Manuals"
LINK

D. Evolution Drives All Cosmic Faring Programs

Dennis Hollenberg (comment 2010-03-31 In A above), you're not going to like the following answer, as unavoidable as it ultimately is:

All spin arrays, regardless of size, temporarily constrained energy packages such as black holes and biospheres, all energy-storing mass-formats, are precariuosly forming and "doing best" to survive "as long as possible", to avoid their energy content being spent on the ongoing fueling of cosmic expansion.

Dov Henis
(Comments From The 22nd Century)
"Gravity Is The Monotheism Of The Cosmos"
LINK

True, genes control/regulate NOTHING; but RNAi is NOT the sole controller/regulator of cells!
by DENNIS HOLLENBERG

[Comment posted 2010-03-31 17:33:57]
The answer to the question "What controls or regulates the global functioning of the cell or developing organism?" is not a gene or coding genes or the genome. Nor is control/regulation restricted to RNAi and ncRNAs. (There is no Santa Claus, and you're not going to like the following answer, as unavoidable as it ultimately is.)

The reason is nature doesn't do "control" or "regulation" in the way that toilers in this field control their TVs, cars, ipods and other techno gadgets. That control-architecture paradigm has been misapplied since pretentious post-Darwinian biologists anointed themselves Oracles of Nature and corbeled together the obviously-brain-dead Modern FrankenSynthesis.

Nature is, after all, seriously in for the long haul and would never stoop to such primitive (read: STUPID) methods for controlling life. Indeed, that the biology business has managed to avoid the truth for all these decades is truly a monument to intellectual lassitude, bureaucratic form-over-content manuscript refereeing ("I haven't seen this viewpoint in the National Enquirer yet, so it must be wrong") and institutional ignorance.

As I have been writing these past decades, the secret door to the answer of how the unfolding organization of life's entities occurs happens to lie, should your walk take you through a relatively unmanaged landscape, under your nose. It is the ecological dynamic. (I warned that you wouldn't like the answer, unavoidable as it is!) The key to that door is here (This paper was banned in the US):

Hollenberg "On the evolution and dynamics of biological networks" _Biologia di Revista/Biology Forum_ 100(1): 93-118 (2007). (Bonus: It's written in English and takes less time than what you spend watching your fav TV sitcom or drinking a glass of chablis.)

Those comfortable with the conventional answers may also wish to acquaint themselves -- thoroughly -- with somewhat more obscure literature:

Darwin _On the origin of the species_ 1859, e.g., NY, Random House (1993).