playing by miR

microRNA research is bringing new insights into cancer pathogenesis. Will it also lead to new strategies for treating cancer? The possibilities are excellent.

BY DARRELL E. WARD

PHOTOGRAPH BY ROMAN SAPECKI

It was 2001 and Carlo M. Croce and his colleagues had searched seven years for a tumor suppressor gene that they were convinced lay at 13q14. That genomic address locates a deletion in chromosome 13 that occurs in more than half of B-cell chronic lymphocytic leukemia (CLL) cases, about half of mantle cell lymphoma cases, 16 to 40 percent of multiple myeloma cases and 60 percent of prostate cancers.

"It is often the only chromosomal abnormality present in CLL, which strongly suggests it is the site of an important tumor suppressor gene," says Croce, MD, now a researcher at Ohio State University Comprehensive Cancer Center (OSUCCC) and director of OSU's Human Cancer Genetics program.

Laboratories around the world had looked too, and although eight new genes were identified in the region, there was no evidence linking any of them to cancer.

Most CLL patients have an indolent form of the disease and survive 10 to 20 years; the rest have aggressive disease that requires immediate therapy and can be fatal in three years, Croce says. Patients with the deletion tend to have the indolent form, but there was no way to know for certain which CLL an individual has at diagnosis.

Croce and his group were stymied in their hunt for the tumor suppressor gene, until autumn 2001 when the journal *Science* published three back-to-back papers showing that microRNA (miRNA)—a novel family of RNA molecules that arise from junk DNA and are far too small to code for a protein—is widely found in worms, flies and humans.

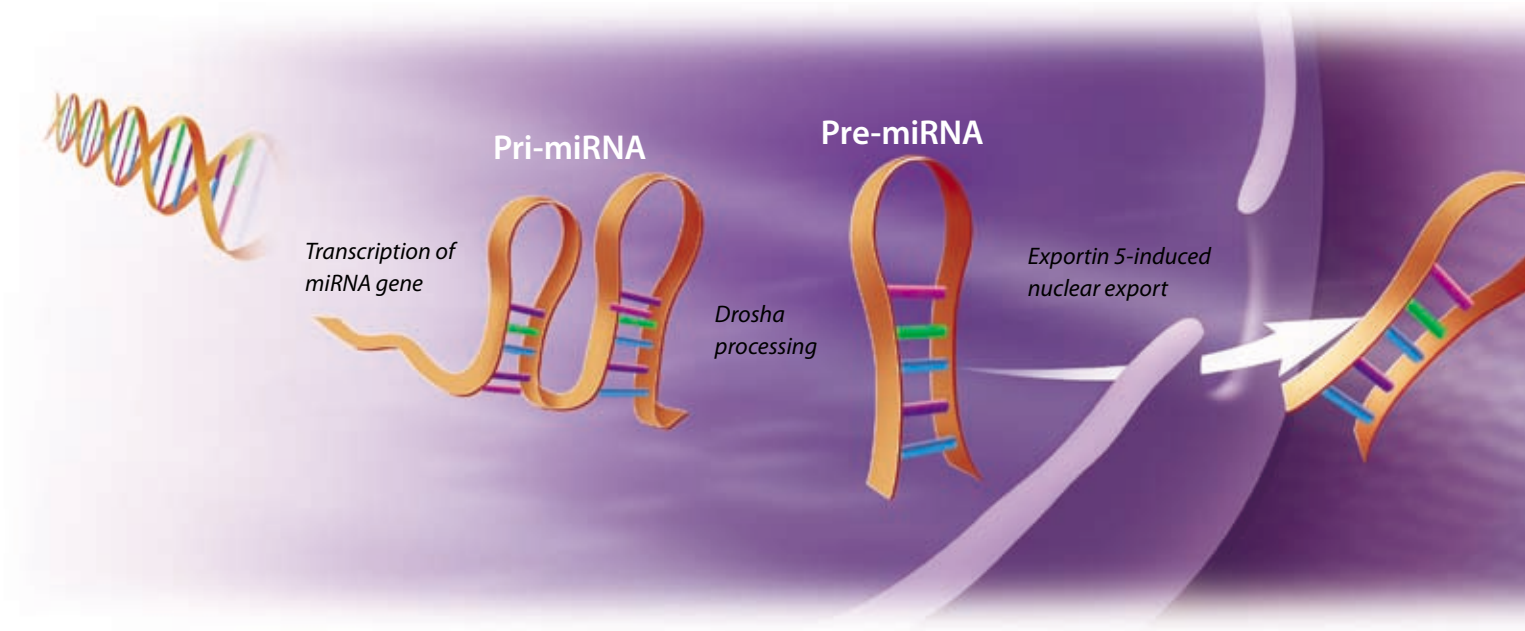
Croce and his colleagues quickly looked again at the region of the deletion, and in a fragment of that fragment they discovered genes for miRNA-15a (MIR15A) and miRNA-16-1 (MIR16-1). "We then looked at CLL cells from 60 patients and found that both these miRNA genes were lost in 68 percent of them," says Croce, who is also the John W. Wolfe Chair in Human Cancer Genetics.

The finding, published in the *Proceedings of the National Academy of Sciences* (PNAS) in 2002, was the first to link miRNA to cancer,

THE FIRST TIME miRNA WAS LINKED TO CANCER WAS IN A STUDY LED BY CROCE AND PUBLISHED IN THE *PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES* IN 2002.

CARLO CROCE, MD

The John W. Wolfe Chair in Human Genetics, professor and chair of Molecular Virology, Immunology and Medical Genetics, director of OSU's Human Cancer Genetics Program



and it changed science's perception of the disease.

"Until then, we thought of cancer as a disease caused by alterations in protein-coding genes," he says. "But there were no protein-coding genes in this very small region that is deleted in CLL—only two miRNA genes. That gave us the first clue that miRNA could be involved in cancer."

miR POTENTIAL

Research by OSUCCC investigators and others has shown that miRNAs act as tumor suppressor genes and as oncogenes, with the same miR sometimes serving as a tumor suppressor in one tissue and as an oncogene in another.

"This is an exciting field because there is a good possibility we can use miRNAs or anti-miRNAs for therapy," Croce says. "Their small size means that they can be readily synthesized in the laboratory and taken up by cells."

MicroRNA research at Ohio State brings together basic and clinical investigators at the OSUCCC and the College of Medicine with investigators in Pharmacy, Veterinary Medicine and Chemistry.

Their studies include the molecular mechanisms and pathways involved in microRNA gene changes in cancer, the basic chemistry and biochemistry of miRNA molecules, and the pharmacology and formulation of miRNA agents for preclinical and pharmacologic studies of formulations of miRNA agents for preclinical and clinical studies of miRNA in diagnosis and cancer therapy.

CANCER LINKS GROW

Croce's 2002 study also showed that MIR15A and MIR16-1 are highly expressed in normal B cells, indicating their importance for maintaining cell health. In a genome-wide bioinformatics search for miRNA genes in 2004, Croce and his colleagues found that about half of the 186 human microRNA genes known at the time lay in regions frequently involved in cancer—fragile sites, break points, and areas that are often amplified or show loss of heterozygosity—further evidence of the molecules' pathogenic role.

The same year, Croce and his colleagues developed a microarray with 245 human and mouse precursor and mature miRNA probes. They used the array to discover that miRNA expression changes correlated with the gene changes that clinically predict CLL progression (e.g., *ZAP70* expression, 13q14 deletions), suggesting that miRNA expression patterns influence CLL biology and behavior.

In a *New England Journal of Medicine* paper in 2005, the researchers identified a signature of 13 miRNAs that reliably distinguished between indolent and aggressive CLL.

"If verified, this signature could determine whether a CLL patient needs very aggressive treatment or not."

Importantly, Croce adds, "for the first time, we found mutations in miRNA genes, both germline and somatic. The germline mutations could be involved in initiation."

They may also offer a new mechanism for cancer predisposition and an explanation for the 10 to 20 percent of familial CLL and other cancer cases for which no predisposing mutations have been found in protein-coding genes, he says.

The researchers found the mutations after sequencing 42 miRNA genes from 75 CLL patients. They identified five mutations in 11 of the patients. "This was fascinating because the mutations are not in the mature miRNA, which is only 21 to 22 nucleotides in length; they are in the precursor, and they probably alter how the precursor is processed."

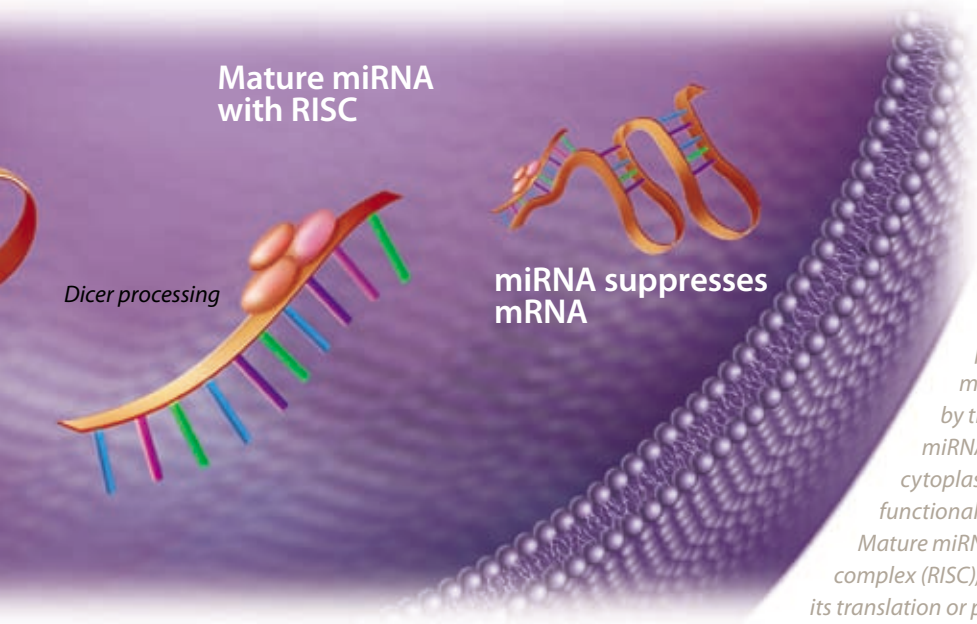


ILLUSTRATION BY THOMAS GRAVES

PRI- BEFORE PRE-

MiRNA is first expressed as a gene transcript called primary miRNA (pri-miRNA). These hairpin-loop molecules remain in the nucleus where they are cleaved by the Drosha complex to form double-stranded precursor miRNA (pre-miRNA). Pre-miRNA is transported to the cytoplasm where it is cleaved by the Dicer complex to form functional, mature miRNA that are about 22 nucleotides long. Mature miRNA first bind with the RNA interference silencing complex (RISC), then with the target messenger RNA (mRNA), blocking its translation or prompting its degradation.

Two patients had germline mutations located at the same site in *MIR15A* and *MIR16-1*. Cheek cells in the patients had both the normal gene and the abnormal gene, but their leukemia cells had only the abnormal gene. "That is a strong indication that these miRNAs are tumor suppressor genes, and that miRNA mutations may be a predisposing factor in CLL," Croce says.

In another 2005 study, Croce and his collaborators determined that MIR15A and MIR16-1 target *BCL2*, a gene that inhibits apoptosis. Low levels of the two miRs correspond to high levels of BCL2. "*BCL2* is over-expressed in most B-cell CLLs," Croce says, "so restoring these miRs may provide a way of treating CLL cases in which *BCL2* is over-expressed."

MIR29

MIR29 occurs in two gene clusters, one on chromosome 1, the other at chromosome 7q32. "We felt the 7q cluster to be particularly important because it is consistently lost in some acute myeloid leukemias," Croce says. *MIR29* is also lost in prostate cancer, lung cancer and a subset of breast cancers.

The cluster consists of three subtypes, *MIR29A*, *MIR29B* and *MIR29C*, which arise through different editing of the same pre-miRNA, he says.

Croce's lab and others had shown that the *MIR29* cluster is down-regulated in non-small-cell lung cancer (NSCLC). The miRs were predicted to target two key DNA methylation enzymes, DNA methyl transferase-3A and -3B, which are often over-expressed in lung cancer and associated with a poor prognosis.

A 2007 study led by Croce showed that expression of the two enzymes in cell lines and NSCLC tissues is inversely correlated with *MIR29* expression, and that the miRs directly target the two methylation enzymes, inhibit cell growth and induce apoptosis.

The researchers showed that forced expression of *MIR29A*, *MIR29B* and *MIR29C* leads to the re-expression of tumor suppressor genes such as *WWOX* and *FHIT* and triggers cell death by apoptosis in lung cancer cell lines, and reduces the size of engrafted tumors by 56 to 94 percent compared with controls.

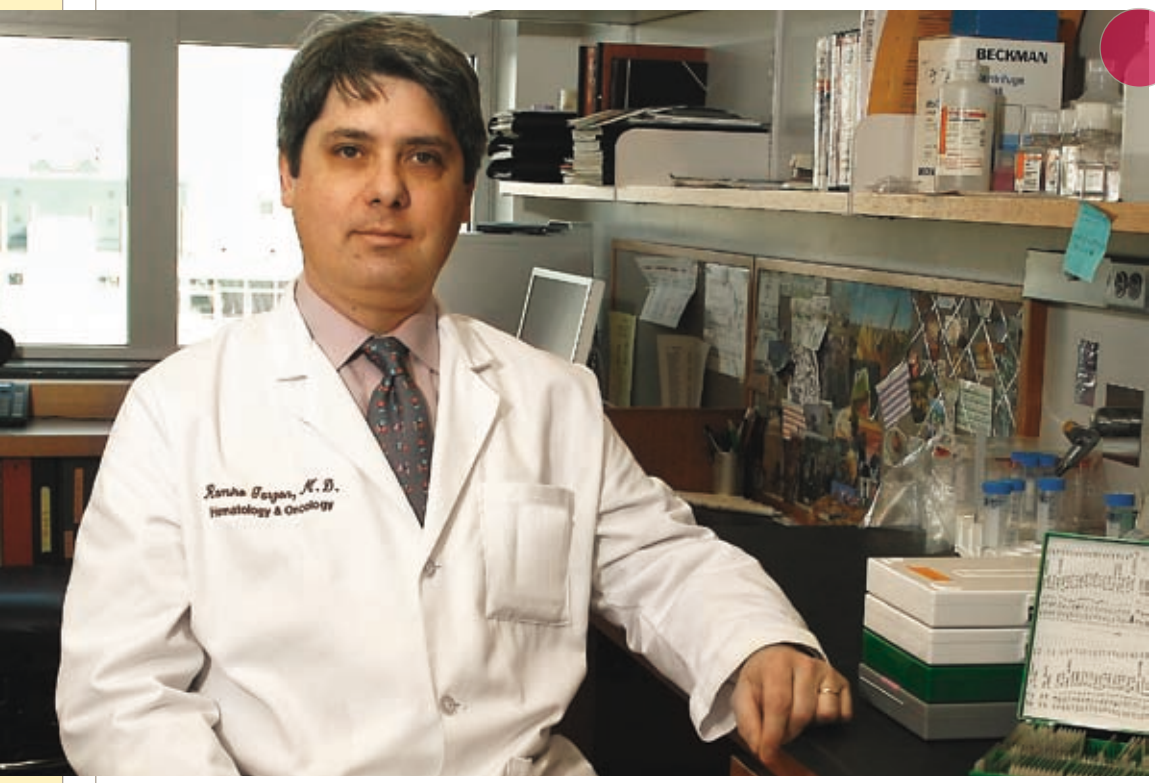
"This showed a new mechanism through which microRNA regulates gene expression," says Muller Fabbri, MD, a researcher in Croce's lab and first author of the *PNAS* paper. "It also showed how miRNA expression can have indirect anti-tumoral effects and why *MIR29* might offer a new therapy for cancer."

THE RESEARCHER

DENIS C. GUTTRIDGE, PhD
OSUCCC Molecular Biology and Cancer Genetics Program

MIR29 AND MUSCLE

One floor below Croce's lab in Ohio State's new Biomedical Research Tower is the laboratory of OSUCCC researcher Denis C.



THE RESEARCHER

RAMIRO GARZON, MD
Hematologist and Oncologist and OSUCCC researcher

Guttridge, PhD, who studies muscle-cell changes in cancer wasting and other diseases and the role of the transcription factor NF-κB in muscle cell differentiation.

Guttridge and his research fellow Huating Wang, PhD, in collaboration with Croce's laboratory, had shown that NF-κB works in conjunction with a protein called YY1 to epigenetically silence a set of muscle-specific genes.

Guttridge and Wang next learned that NF-κB, in conjunction with YY1, also epigenetically silences *MIR29* in immature muscle cells, and that *MIR29* promotes muscle cell differentiation.

"At that point Carlo and his lab joined us and took the study to a new level," Guttridge says. "We then asked if MIR29 functions as a tumor suppressor in muscle."

Guttridge turned to the nearby Research Institute at Nationwide Children's Hospital, which has the nation's only recognized rhabdomyosarcoma (RMS) tumor bank. The Ohio State investigators screened several RMS cell lines and ten RMS tumors and found that *MIR29B* expression was down by 90 percent or more in both compared with controls. When they forced the expression of *MIR29B* in an RMS cell line, cell growth slowed more than two-fold, and the cells began forming multinucleated myotubes.

"Our study indicated that *MIR29B* is a tumor suppressor in rhabdomyosarcoma, but that, in skeletal muscle, it functions by promoting differentiation rather than by causing cell death," Guttridge says.

"It also suggests that inhibiting NF-κB or intervening at the microRNA level might offer new therapeutic approaches for rhabdomyosarcoma."

AML

Early in 2009, OSUCCC investigators Guido Marcucci, MD, associate professor of Internal Medicine; Clara D. Bloomfield, MD, Distinguished University Professor; Croce and Ramiro Garzon, MD, assistant professor, reported findings showing that MIR29B not only targets DNA methyltrasferase-3a and -3b in AML, but also DNA methyltransferase 1 (*DNMT1*).

"We showed for the first time in leukemia cell lines and patient samples that over-expressing *MIR29B* decreases DNA methylation, re-expresses the tumor suppressor genes p15 and *ESR1* and induces blast-cell differentiation," Garzon says.

A second study explored how MIR29B functions as a tumor suppressor, showing that restoring *MIR29* expression causes apoptotic cell death. "We learned that MIR29B targets four major pathways that are affected in leukemia, those involved in apoptosis, cell death, proliferation and epigenetic silencing of tumor suppressor genes," Garzon says.

miRNA AS THERAPY

Studies of synthetic MIR29B and MIR16-1, and an anti-miRNA (or antagomir) as agents for the treatment of CLL, AML, NSCLC and hormone-resistant breast cancer are being done by a group of OSUCCC clinical and translational researchers, and drug development specialists.

"It's a challenge to deliver miRNAs into cells," says drug delivery specialist Robert J. Lee, PhD, associate professor of Pharmaceutics and a researcher in the OSUCCC Experimental Therapeutics Program. "We've developed nanoparticle formulations that seem to work, but it's too early to say that we've found the best delivery system."

Lee and his colleagues work closely with Ohio State's Nanotechnology Center to package synthetic MIR29B in nanoparticles that carry an antibody or ligand on their surface that targets tumor cells and

triggers their uptake by endocytosis (see illustration).

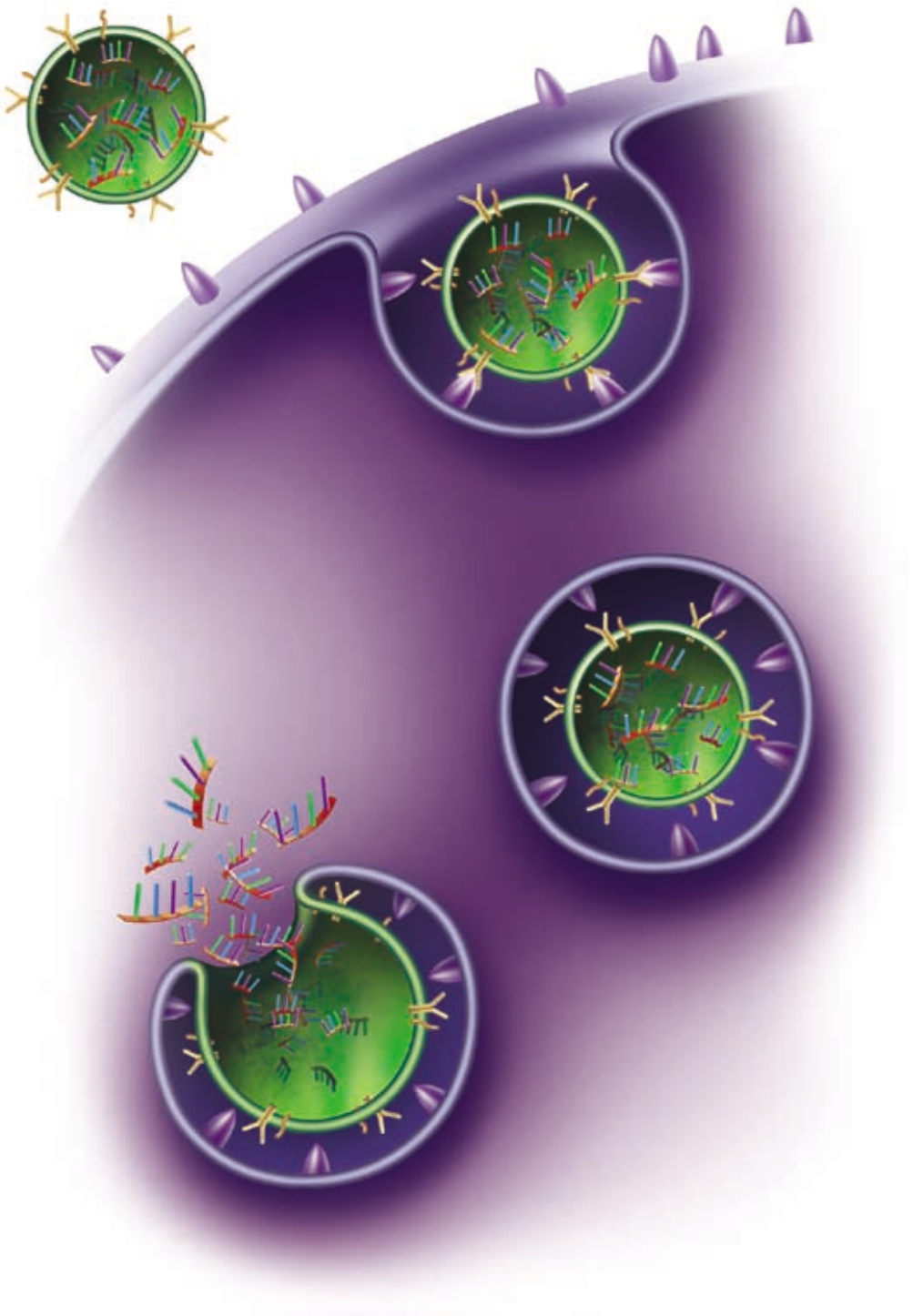
Pharmacokineticist Kenneth K. Chan, PhD, professor of Pharmaceutics and of Internal Medicine and a researcher in the OSUCCC Experimental Therapeutics Program, conducts pharmacokinetic studies of the prospective agents. He developed a method called hybridization-based fluorescent ELISA for measuring exogenous miRNAs in cell lysate and circulating blood at picomolar levels.

"Because miRNAs will likely be used in combination with other drugs or with other miRNAs, we have also developed methods that allow us to measure an individual miRNA in a mixture of several miRNAs," Chan says.

MiRNAs present fascinating questions. "MiRs seem part of a major puzzle that we are just starting to decipher," Fabbri says. "We still lack the whole picture of the miRNA pattern in a tumor. We don't know, for instance, if they interact with each other, or if they require certain conditions to interact with a target that are present in some tumors and not in others."

This could explain the cancer-specific differences in miRNA behavior, and why computationally and biostatistically predicted miRNA targets can be experimentally validated only part of the time, he notes.

Among the most important questions is whether miRNA agents offer safe, effective therapies. "We are doing studies now to learn if synthetic MIR29B can achieve therapeutic concentrations and effectively down-regulate target genes in animals," Garzon says. "Perhaps then we can begin human trials."



GOOD THINGS IN SMALL PACKAGES

OSUCCC investigator Robert J. Lee, who specializes in receptor-targeted drug delivery systems, is testing the use of nanoparticles to carry therapeutic MIR29B to acute myeloid leukemia (AML) cells. A lipid-polymer shell encloses the microRNA and displays CD33, a monoclonal antibody specific for AML cells. Binding of the antibody with the cell receptor triggers the endocytotic uptake of the particle and the intracellular release of the miRNA.

ILLUSTRATION BY THOMAS GRAVES