Tiny Yet Powerful

microRNA (miRNA) are one of a group of small, noncoding, regulatory RNA; approximately 22 nucleotides (nt) in length, they regulate posttranscriptional gene expression by repressing or breaking down messenger RNA (mRNA). They occur in plants, invertebrates, and vertebrates, including humans. In a commentary in *Cell* last year, Gary Ruvkun, Bruce Wightman, and Ilho Ha described the technical roadblocks they and colleagues have faced along a 20-some year journey of identifying miRNA and determining their function. Over time, the discovery and development of molecular techniques have revealed increasing numbers of miRNA families and are allowing researchers to identify their target genes, as well as the mechanism by which they are processed from larger precursor transcripts to their tiny, functional selves.

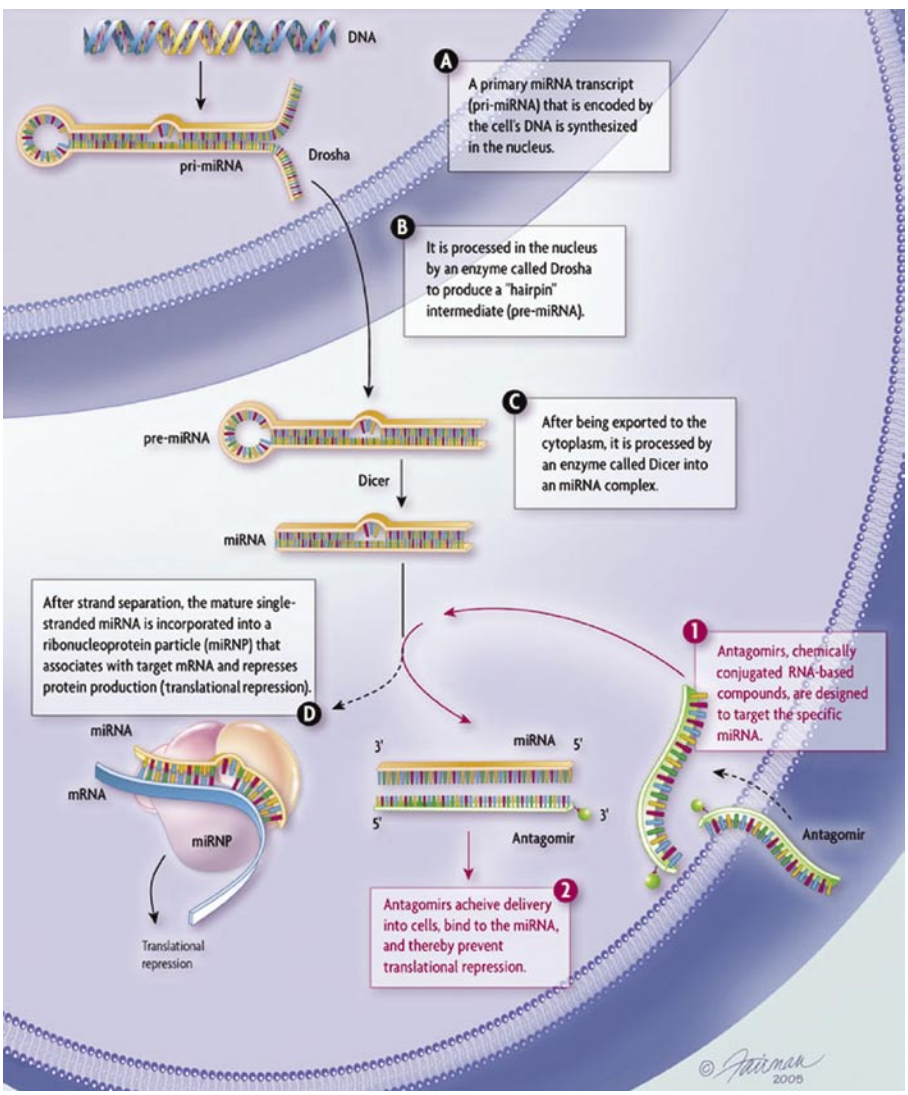
Small Worms, Abundant Information

The existence of a detailed analysis of *Caenorhabditis elegans* cell lineage and developmental genetics, along with a collection of mutants, including some that caused developmental timing defects, played a role in identifying the first miRNA. Victor Ambros was working on *lin-4*, a negative regulator of developmental timing, in Robert Horvitz' laboratory, while Gary Ruvkun was working on *lin-14*, a heterochronic gene, which temporally regulated the fate of cells during larval development. *lin-4* encoded an RNA but did not appear to have an open reading frame. *lin-14* looked like a protein-coding sequence (in fact, it encodes a novel transcription factor) with a long 3′ untranslated region. They eventually discovered that *lin-4* RNA was complementary to a region in the *lin-14* 3′ region, which suggested that *lin-4* might be binding to *lin-14*. *lin-4*, the first known miRNA, progressively represses the translation of *lin-14* mRNA, the first miRNA target. Subsequently, Frank Slack, Associate Professor, Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT, joined Ruvkun's lab and worked on *let-7*, which was the second miRNA and which, like *lin-4*, is involved in developmental timing. Although in the same pathway as *lin-4* and *lin-14*, conservation of the *let-7* sequence in other organisms, including fruit flies and other invertebrates and humans, suggested that this system had wide-reaching importance beyond *C. elegans*.

In animals, there may be sequence differences of seven to eight or more nucleotides between an miRNA and its targets, making target prediction difficult. In addition, a single miRNA could have several to hundreds of different targets. Slack says there may be 120 to 500 miRNA in *C. elegans*, "depending on who you believe. A lot is known about *let-7*, and there are validated targets in *C. elegans*." Slack suggests that looking for conserved regions can allow one to design rules to identify new targets using a bioinformatics approach, "although not every miRNA works the same." The rules can be validated in *C. elegans* before the human or other homologues are examined. *C. elegans* shares 60% of genes with humans, and there are at least 30 to 40 *C. elegans* miRNA conserved in other organisms that have been around since the last common ancestor of *C. elegans* and the mouse.

Looking at Trees

miRNA in plants differs from those in other classes of organisms in key ways. Vincent Chiang, Professor, Department of Forestry, North Carolina State University, Raleigh, points out that although in animal systems one may detect both the precursor transcript and the mature miRNA, in plants, for reasons that are unknown, the precursor is transient, and only the mature 21- to 24-nt-long miRNA is detectable. Also, in plants, miRNA have almost perfect

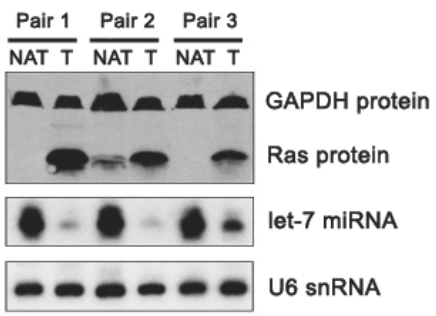


Courtesy of Muthiah Manoharan, Alnylam Pharmaceuticals, and Markus Stoffel, Rockefeller University.

Mechanism of antagomir action.

complementarity to their targets, so it is easier to predict targets through a computational approach. Chang notes "hybridization methods (e.g., microarrays and Northern blots) detect collective expression of an miRNA family, but you can't tell which family member contributes the most to expression." He and colleagues have developed a real-time PCR technique that detects mature miRNA in plants. "In principle, it is simple: add a poly(A) tail to make small sequences longer, then treat the longer molecule as usual in a quantitative PCR assay. Our technique varies the annealing temperature of the PCR, so it can distinguish miRNA differing by only one nucleotide in the sequence. It is very useful to determine quantitative expression of individual miRNA and can differentiate the most expressed family member. I think this is a very important step in the linear path from discovery to functional analysis," he concludes.

The existence of many *Arabidopsis* mutants created for other purposes may be useful for determining which miRNA targets a particular mutated gene in this species. "Conservation is another issue," says Chiang, whose work focuses on trees. The poplar, *Populus trichocarpa*, is the only tree species with a fully sequenced genome. Half of the poplar miRNA are not present in *Arabidopsis*. Similarly, 50% of human miRNA are not conserved in other animals. Chiang expects even more species-specific miRNA will be identified as techniques continue to become more sophisticated. Slack concurs that miRNA have to be expressed at high enough level to be detectable. "If an miRNA is only in one cell type at a certain point in development, you won't see it unless you're looking for it."



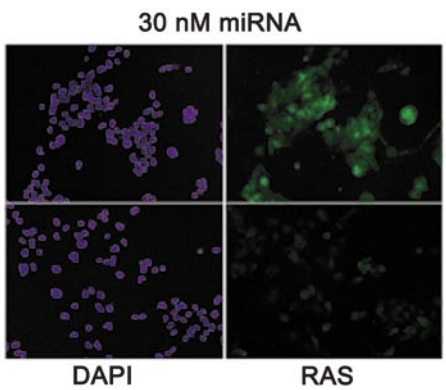
Courtesy of Frank Slack, Yale University.

When *let-7* miRNA levels are low in lung tumor samples (T), protein levels encoded by the oncogene *RAS* are high. In normal adjacent tissue (NAT), *let-7* miRNA levels are high, and RAS protein levels are low. GAPDH, glyceraldehyde 3-phosphate dehydrogenase (protein standard); U6 snRNA, small nuclear RNA U6 (RNA standard).

In trees, where there are no transgenic or mutant organisms, functions of miRNA are being predicted from the function of their target genes. The ones that have been identified are largely related to disease resistance (e.g., crown gall disease in pine, a species important for pulp and paper production). miRNA may also be involved in tree responses to mechanical stress, such as wind force and snow weight, as well as to the load pressure exerted by their own crowns.

More Mouse and Man

Markus Stoffel, Robert and Harriet Heilbrunn Professor, Rockefeller University, New York, NY, believes that most of the miRNA that are highly expressed have been identified. "The big question," he says, "is, what is their function? For most, it is a big black box, especially in mammals." Doing knockouts in this system is a "slow, expensive process, and tricky. miRNA affect many encoded genes, there is redundancy, they occur on more than one chromosome, some miRNA are intronic, and some are in polycistronic clusters. The precursor is one big transcript that is cut into pieces, so it's hard to knock it out genetically and affect individual miRNA." His group's approach is to silence miRNA using antisense RNA, which they call antagomirs, stabilized by chemical modification to render them resistant to RNases and allow them to enter cells. They have shown that silencing miR-122, an abundant, liver-specific miRNA, appeared to down-regulate cholesterol synthesis genes. "This is proof of principle," Stoffel says. "miRNA look like perfect targets, but we still need to find out what they do. Their role in disease is still not clear, but I would be surprised if miRNA did not play a role in disease."



Courtesy of Frank Slack, Yale University.

Normal *RAS* expression levels in HepG2 cells (upper panels) are repressed in the presence of *let-7* miRNA (lower panels). Left panels, 4′,6-diamidino-2-phenylindole (DAPI) staining for DNA; right panels, detection of RNA with anti-RAS antibody.

Future Directions

Chiang is looking at miRNA to understand how wood is formed in trees. With only one tree genomic sequence complete, "the pine people are working on getting support for sequencing for that species," he says. "They are hoping for an industry and government collaboration for more resources, because the pine genome is 50 times bigger than the human genome." He and collaborators are working on pine genomics based on expressed sequence tag (EST) sequences. There are about 400,000 ESTs available, although "they are not ideal to discover miRNA without a fully sequenced genome," he says, "so the field is moving slowly." Only eight or nine of the hundreds to thousands of plant miRNA have a proven function, so there is plenty to be discovered.

Slack expects miRNA will eventually play a role in curing or at least alleviating symptoms of cancer and that this will draw on experience with both antisense and small interfering RNA (siRNA) techniques. Because miRNA are in essence natural products of the cell, "using miRNA to usurp the natural process instead of forcing unnatural antisense molecules" to do so will be more effective, he predicts. "miRNA microarrays are so hot because miRNA by nature are regulatory molecules, present in discrete places at discrete times. So, miRNA arrays may be better diagnostic tools to detect tissue types than standard microarrays, for example, to determine the tissue type of a metastatic tumor." Slack points out that in the early days of the antisense and monoclonal antibody revolution, there was excitement about their potential to treat many diseases. "Few therapeutics in these classes have made it into the clinic, but those that have are billion dollar drugs. Few miRNA-based therapeutics will probably make it. In 10 years, we may see the first drugs; in 20 years, they will be more common. However, the field changes so fast they might be successful earlier, or we may find they cause side effects." This is because miRNA, at least in animals, target many genes. Therefore, rather than use one miRNA to knockout one gene, one might be able to identify patients whose disease is due to deletions or low expression of miRNA resulting in altered regulation of the many different genes it controls. Whether miRNA will play a role in diagnostics, therapeutics, or possibly even gene therapy, remains to be seen.

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