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## Tapping miRNA-Regulated Pathways

### Expression Profiling Ramps Up to Support Diagnostics and Drug Discovery

Vicki Glaser

miRNAs are master regulators of gene expression, according to William S. Marshall, Ph.D., president and CEO of **miRagen Therapeutics**. "You can have one microRNA that controls multiple genes and one gene that is controlled by multiple microRNAs." They exert negative regulation and have been shown to control expression of entire signaling pathways.

miRNA discoveries appear in the news on a regular basis. For example, a research team at the **Wistar Institute** recently reported the identification of two miRNAs—miR-373 and miR-520c—that are members of the same miRNA family and were shown to promote tumor metastasis. The two miRNAs are not found in normal adult cells, only in tumor cells, according to the authors. Of the 450 miRNAs tested, these two, described by Tony Huang, Ph.D., and colleagues, induced cell migration in the MCF-7 line of human breast cancer cells, which normally do not metastasize. They could serve as biomarkers for the metastatic potential of breast cancers and the need for more aggressive treatment.

As the number of miRNAs identified continues to grow, researchers are exploring the biology of miRNA function and characterizing the tissue specificity and range of activity of individual miRNA molecules. Changes in miRNA levels have been correlated with disease processes. A great deal of work is under way to study the effects of over- or underexpression of specific miRNAs on the development and inhibition of pathogenesis, particularly in the areas of cancer, as well as in heart disease, neurological disorders, and aging.

Researchers in industry and academia will present their latest findings at CHI's "microRNA in Human Disease and Development" meeting to be held later this month.

"The microRNA expression profile is extremely indicative of cell type," continues Dr. Marshall. "When a cell transforms into a disease state, there is typically a perturbation in the microRNA level." Approximately 400 to 500 miRNAs have been characterized in humans to date, according to Dr. Marshall, and 80–150 are typically expressed in any particular cell type.

miRagen, which will focus on the role of miRNAs in cardiovascular health and disease, is a newly established company based on research being conducted in the laboratory of Eric Olson, Ph.D., chair of the department of molecular biology at **University of Texas Southwestern** and cofounder.

#### Decoding a Complex Picture

"We believe that individual microRNAs regulate the expression of tens, if not hundreds, of independent genes," and many of those affect related pathways and overlapping processes, says David Brown, Ph.D., director of R&D at **Asuragen**. "Our studies of microRNAs indicate that a single, small RNA often regulates a given cellular process by affecting the expression of genes in two to four related pathways."

Researchers at Asuragen, in collaboration with the laboratory of Frank Slack, Ph.D., at **Yale University**, have shown that the let-7 miRNA is expressed in normal lung tissue and that inhibition of let-7 function leads to increased cell division in lung cancer cells. The let-7 controls cell-cycle progression, and overexpression of let-7 in cancer cells can reduce cell division. The let-7 family of miRNAs has been shown to repress multiple genes involved in the

cell cycle and cell division, including RAS.

At the Computational Biology Center of **IBM's** Thomas J. Watson Research Center, Isidore Rigoutsos, Ph.D., manager of the bioinformatics and pattern-discovery group, and colleagues are studying miRNA and its effects on biologically significant transcripts. The work involves applying a pattern-based method for identifying miRNAs as well as miRNA-binding sites and their corresponding heteroduplexes. It is demonstrating that—in addition to the proposed mechanistic model for animal miRNA function, by which miRNAs act through the 3' untranslated regions (UTRs) of targeted transcripts—animal miRNAs may also extensively target sites in amino acid-coding regions and 5' UTRs.

Dr. Rigoutsos' group has argued that based on computational analysis there may be as many as 50,000 miRNAs in the human genome and each may have as many as a few thousand potential targets. To support the contention that miRNAs such as miR-134, which has been implicated in embryonic stem-cell differentiation in mice, may have numerous targets, Dr. Rigoutsos and colleagues randomly selected 158 of the roughly 2,300 predicted 3' UTR targets of miR-134's. Using a luciferase assay, they demonstrated greater than 30% suppression of luciferase activity in 129.

The conclusion that one can draw from these findings is that the extent of miRNA activity and the mechanisms by which they regulate gene expression “are likely more complicated than previously acknowledged,” says Dr. Rigoutsos. Furthermore, “There is accumulating evidence that short RNAs can not only affect the levels of proteins, but that proteins may also affect the production of microRNAs.”

Asuragen is applying miRNA biomarkers in the development of molecular diagnostic assays with a primary focus on cancer. As evidence mounted that “the altered expression of a single microRNA profoundly affects critical regulatory pathways in humans,” Asuragen expanded its focus to explore their potential therapeutic applications, explains Dr. Brown. The company has been using cell and animal models to explore the concept of miRNA-replacement therapeutics, in which the reintroduction of an miRNA in a diseased cell would reinitiate pathways that have been turned off by the downregulation of the miRNA.

The literature includes examples of miRNAs that function as oncogenes, with their overexpression contributing to tumorigenesis, and of others that act as tumor suppressors, which when downregulated contribute to cancer. Asuragen is focusing on miRNAs that act as tumor suppressors and is using synthetic versions of a few key miRNAs to reduce or eliminate the expansion of cancer cells in vitro and in vivo.

Changes in expression of a single miRNA have been implicated in some cancers, and “downregulation of microRNAs is critical for the maintenance of cancer cells,” says Dr. Brown. Reintroduction of miRNAs in animals has been shown to impair the viability of cancer cells and result in cell death. Asuragen expects to advance one or more miRNA therapeutic compounds from feasibility testing to preclinical studies within the next year.

The company is currently launching a commercial venture called **Mira Therapeutics**, which will enable an expanded effort to develop miRNA-based drugs.

If a single miRNA can have a role in regulating multiple pathways, would miRNA replacement therapy carry a risk for off-target effects? “We truly believe there are no such things as off-target effects for microRNAs,” says Dr. Brown. “We are not introducing anything that is not already present in the cell.” Throughout evolution, the biology of mammals has been geared to regulation by miRNAs, and miRNA-replacement therapy would simply reintroduce an miRNA that would normally be present, Dr. Brown contends.

Mike Wilson, Ph.D., array R&D manager at Asuragen, will describe the company's molecular diagnostics development effort in a presentation entitled, “microRNA Expression Profiles Associated with Colorectal Cancer and Derived from FFPE Tissues.”

### Therapeutic Targets

Eugenia Wang, Ph.D., professor at the University of Louisville, has proposed that miRNAs have a critical role in “a universal or system-specific programmatic shift of signaling control” that occurs at mid-life and brings about a decline in cellular health status associated with aging, which may precipitate increased risk of late-life diseases. In her presentation, she will review the hypothesis that the changes in expression of most if not all aging-related genes are controlled by underlying hubs and the belief that miRNAs, acting as molecular master switches, are candidate hubs.

Dr. Wang’s research has focused on two distinct systems, the aging mouse liver and peripheral lymphocytes from individuals with sporadic Alzheimer’s disease. She is using microarray-based screening to profile changes in age- and disease-related miRNA expression.

Dr. Wang has developed miRNA microarrays called MMChips that contain all known miRNAs for a particular species. To date, these include seven different MMChips—human, mouse, rat, dog, *C. elegans*, *Drosophila*, and a microarray targeting viral-specific miRNAs. Using these in parallel with tandem mass spectrometry-based proteomic-profiling techniques, Dr. Wang performs comparative mapping of up- and downregulated miRNA expression and associated down- and upregulation of specific proteins.

By isolating miRNAs from the livers of young, middle-aged, and old mice, Dr. Wang can look for changes in miRNA levels as the animals age. She has shown that an organism’s maintenance microRNAs—those responsible for fine-tuning its cell state and behavior, including regulators of the cell-DNA cycle, DNA repair, oxidative stress responses, and apoptosis—start to become abnormally expressed in midlife, which causes deterioration in six or seven general pathways.

Dr. Wang’s work in sporadic Alzheimer’s disease focuses on the role miRNAs might have in the systematic deterioration that can lead to age-dependent diseases such as cardiac disease, osteoporosis, and Alzheimer’s. The studies have identified three miRNAs that are upregulated in patients with sporadic Alzheimer’s disease compared to age-matched controls. These miRNAs are involved in cell-cycle regulation.

Eva van Rooij, Ph.D., a postdoctoral researcher in the department of molecular biology at the UT Southwestern Medical Center, notes that “microRNAs act upstream of disease processes and so are powerful regulators of downstream cascades. They tend to regulate groups of genes with common functions such as cell growth or survival. microRNAs are also adept at regulating important signaling pathways.” In a talk entitled “The Myriad Roles of microRNAs in Heart Disease,” Dr. van Rooij describes her ongoing work to characterize the effects of manipulating stress-responsive miRNAs on cardiac muscle disease.

miRNAs have been implicated as positive and negative regulators of growth, development, function, and stress responsiveness of the heart. A group of stress-responsive miRNAs including miR-195, miR-208, and miR-21 are up- or downregulated during pathological cardiac remodeling and may play a role in the development of cardiac hypertrophy and heart failure.

Dr. van Rooij’s work focuses on miR-208, which is only expressed in the heart. She is confident that efforts to develop an inhibitor of miR-208 will allow for tissue-specific downregulation.

To understand the biology of miR-208, Dr. van Rooij and colleagues knocked down its expression in mice, stressed the animals’ hearts, and found that the typical response to stress on the heart, characterized by hypertrophy and diminished pumping capacity, was not evident.

In the absence of miR-208, the heart was better able to handle the stress. The combination of its cardiac-specific expression and role in the heart’s response to stress led Dr. van Rooij and colleagues to pursue miR-208 as a therapeutic target for heart disease.

### **Enabling miRNA Research**

**Exiqon** recently announced that it plans to merge with **Oncotech**, a developer of cancer

diagnostics, to leverage its miRNA analysis technology. Through the company's cancer testing services, which use its Extreme Drug Resistance (EDR®) assays, Oncotech has "collected a biobank of 150,000 biopsy samples with associated clinical data," says Søren Møller, Ph.D., Exiqon's vp of R&D.

Exiqon intends to develop a series of miRNA-based drug-resistance and treatment-selection tests that will be marketed through Oncotech's CLIA laboratory.

Dr. Møller describes miRNAs as a sweet spot for the company's core locked nucleic acid (LNA) technology due to the challenges inherent in working with these small RNA molecules. LNAs are nucleic acid analogues that provide enhanced thermal stability and target discrimination. One of the challenges in microarray-based miRNA screening is the range of GC content and associated melting temperatures (T<sub>m</sub>) of the hybridization probes.

"LNA technology allows us to T<sub>m</sub> balance all the probes on an array," says Dr. Møller. "And the high specificity of LNA capture probes allows us to differentiate between closely related microRNAs based on a single base mismatch," he adds.

Exiqon has developed detection probes designed for in situ hybridization using tissue or cell specimens to detect the spatial distribution of miRNAs in histology samples. The company recently introduced the miRCURY™ LNA microRNA PCR system for miRNA detection and quantification.

Dr. Møller's presentation at the conference, entitled "microRNA Profiling of Cancer Using a Novel LNA-Based Microarray," describes Exiqon's work on developing global expression profiles of miRNAs in breast cancer and normal adjacent tissue with the goal of identifying diagnostic biomarkers and prognostic signatures. The company has identified numerous, differentially expressed miRNAs including let-7a/d/f, miR-125a/b, miR-21, miR-32, and miR-136 and has confirmed the microarray results using quantitative PCR. It has also identified several miRNA candidates previously not linked to breast cancer using next-generation DNA sequencing technology.

Chris Hebel, director of business development at **LC Sciences**, attributes the company's ability to custom design microfluidic microarrays for miRNA profiling to the flexibility inherent in the technologies it employs to create the arrays. The μParaflo™ Biochip platform relies on three main technologies: microfluidics; a synthesis chemistry that can use standard building blocks to make DNA, RNA, or peptides and can accommodate modified nucleic acids or amino acid analogues; and light deprotection/photolithography to drive the synthesis of the probes directly on the chip.

LC Sciences' synthetic strategy and its ability to produce custom-designed chips offer two main advantages, according to Hebel. It can normalize the T<sub>m</sub> of the probes and achieve uniform binding across an array. It can also modify and add new probes as the database of known miRNAs expands.

The company adopted a service-based business model rather than opting to sell its chips. Hebel has seen the miRNA research market broaden over the past year. Whereas the company's customers initially were primarily focused on cancer and neuroscience research, the customer base has grown to include larger numbers of cardiac researchers, virologists, plant scientists, and cell biologists.

The company has also added related miRNA services such as qPCR and gene expression array-based profiling and continues to maximize the probe density on its chips.



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