Gaetano Santulli   *Editor*

microRNA: Medical Evidence

From Molecular Biology to Clinical Practice

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Foreword

It gives me immense pleasure to introduce *microRNA: Medical Evidence—From Molecular Biology to Clinical Practice* to the medical and scientific community. The book you are holding was developed to provide medical students, researchers, and physicians with the knowledge on an emerging fundamental section of biomedicine: microRNA.

This book represents one volume—focused on clinical practice—of a trilogy exploring the functional role of microRNAs from basic science to the clinical scenario. The other two volumes explore the importance of microRNA in molecular biology and in cancer, respectively. The books have been edited by Dr. Gaetano Santulli, MD, PhD, who reunited the major experts in the microRNA field in order to have a comprehensive, up-to-date, and systematic overview of the mechanistic roles of these tiny molecules in physiology and disease.

MicroRNAs are small endogenous noncoding RNAs (~22 nucleotides) that fine-tune gene expression at the posttranscriptional level through mainly binding 3′-UTR of mRNAs. They are involved in numerous pathophysiological processes within cells and represent major regulators of gene expression by virtue of their preponderance to target transcription factors.

Following an introduction to precision medicine and personalized therapies, the book propones diverse chapters discussing the role of microRNAs in neurologic disorders, including epilepsy, autism, chronic pain, fragile X syndrome, and neurodegenerative disease. Then, a series of chapters extensively describes the clinical aspects of microRNAs in both diagnosis and therapy of metabolic and cardiovascular disorders, focusing on mitochondrial fitness, arterial hypertension, cardiovascular remodeling, cerebrovascular disease, pulmonary hypertension, diabetic kidney disease, and kidney transplantation. In the following chapters the experts discuss the importance of microRNAs in the wound healing process and in skin disease, in the pathogenesis of allergy, in human ovulation, and in infection. An interesting outline on the emerging role of microRNAs in the field of doping and a chapter explaining in detail microRNA profiling conclude the book.

This book highlights the functional roles of microRNAs in various human disorders, discussed in a detailed manner by expert contributors. Worldwide renowned experts also emphasize the current challenges and outstanding questions for the application of microRNA in clinical practice. The book includes many color pictures, schemes, and diagrams that will be very helpful to students and physicians and eloquent tables that support the text.

The clinical profile is evident in each chapter. The authors have done a terrific job in presenting such complex topics in an easy and comprehensible manner.

Milan, Italy   Gianluigi Condorelli

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Chapter 1
Exploiting microRNA Specificity and Selectivity: Paving a Sustainable Path Towards Precision Medicine

Gaetano Santulli

Abstract In his State of the Union address before both chambers of the US Congress, President Barack Obama called for increased investment in US infrastructure and research and announced the launch of a new Precision Medicine Initiative, aiming to accelerate biomedical discovery. Due to their well-established selectivity and specificity, microRNAs can represent a useful tool, both in diagnosis and therapy, in forging the path towards the achievement of precision medicine. This introductory chapter represents a guide for the Reader in examining the functional roles of microRNAs in the most diverse aspects of clinical practice, which will be explored in this third volume of the microRNA trilogy.

Keywords miRNA • Pharmacogenomics • Precision medicine • Initiative • Selectivity • Specificity • Pharmacogenetics

In his last State of the Union address before both chambers of the US Congress, President Barack Obama called for increased investment in US infrastructure and research and announced the launch of an innovative *Precision Medicine Initiative*. "I want the country that eliminated polio and mapped the human genome to lead a new era of medicine—one that delivers the right treatment at the right time," he said. "Tonight, I'm launching a new Precision Medicine Initiative to bring us closer to curing diseases like cancer and diabetes—and to give all of us access to the personalized information we need to keep ourselves and our families healthier," he continued [1].

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Such an announcement offers an illustration of the considerable interest that exists in achieving greater progress in treating disease [2].

President Obama has long expressed a strong conviction that research offers great potential for improving health [3]. One million or more US citizens will be powering President Barack Obama's Precision Medicine Initiative. This bold volunteer-driven move to collect and link genotypic, phenotypic, and lifestyle data, including crowdsourcing and social media tools, aims to accelerate biomedical discovery with an initial focus on cancer [4].

The patient-participant cohort at the core of the initiative will enable new approaches to prevention, diagnosis, and treatments tailored to individual patients. "It's a new model for doing medical research," says National Institutes of Health's (NIH) director Francis Collins, while discussing the precision medicine approach [3, 4].

Do microRNAs (miRs) have a role in precision medicine? The answer is yes, and apparently not only at an interindividual level but also at an intercellular level. Indeed, miRs are exquisite regulators of gene expression that inhibit translation and/or promote mRNA degradation by base pairing to precise complementary sequences within the 3′-untranslated region.

They are expressed in a cell-specific manner and give us the possibility to generate selective treatments that target the bad cells and preserve the good cells, with major implication in cancer (see the second volume of the trilogy, where these aspects are discussed in detail) but also in other disorders [5–14].

This introductory chapter opens the third volume, where miRs will be analyzed in the clinical scenario. In the next years, clinicians will have to deal with miRs, not just as diagnostic biomarkers but also as potential tools to design selective treatments, alongside with their emerging important role in prognostic signatures and prediction models.

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Chapter 2
microRNAs and Personalized Medicine: Evaluating Their Potential as Cancer Biomarkers

Anne Saumet and Charles-Henri Lecellier

Abstract microRNA deregulations are often, if not invariably, associated with human malignancies, including cancers. Though most of these deregulations may not be functionally implicated in tumorigenesis, the fact that microRNA expression can be monitored in a variety of human specimens, including biological fluids, supports studies aimed at characterizing microRNA signatures able to detect various cancers (diagnosis), predict their outcome (prognosis), monitor their treatment (theranosis), and adapt therapy to a patient (precision medicine). Here, we review and discuss pros and cons of microRNA-based approaches that can support their exploitation as cancer biomarkers.

microRNA Biogenesis

The microRNAs (miRNAs) are a class of 18–25 nucleotides long RNAs involved in the repression of translation and in the adjustment of protein production in response to various stimuli [1–3]. Their expression must be accurately controlled to ensure

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plethora of cellular processes [4–6]. The miRNA biogenesis involves several steps, each step being subject to specific controls (for review [7]). Briefly, a long (thousand nucleotides long) RNA called the primary-miRNA (pri-miRNA) is transcribed from the genome mostly by the RNA polymerase II. This pri-miRNA contains one or several local stem-loop structures (called precursor(pre)-miRNA) in which the mature miRNA sequence is embedded. Next, a specific complex, called the Microprocessor and containing the RNAse III Drosha, crops the pre-miRNA from the pri-miRNA. The pre-miRNA is exported to the cytoplasm where another RNAse III, Dicer, processes the pre-miRNA into duplex of miRNAs. Only one strand of this duplex will guide a protein complex onto mRNAs harboring partial sequence homology and eventually trigger translation repression mostly by mRNA exonucleolytic cleavage [8]. The first two steps are believed to be the main control points for miRNA regulation [7, 9]. Similarly to protein coding genes (PCGs), control of pri-miRNA transcription involves DNA-binding proteins (i.e., transcription factors, TFs) that recognize specific *cis*-regulatory DNA motifs in the promoter region of the pri-miRNA. The definition of miRNA promoters remains elusive. The pri-miRNAs are unstable molecules making hard the precise identification of their 5′ end, i.e., miRNA Transcription Start Sites (TSSs). Numerous studies have tackled that problem and proposed different approaches to characterize miRNA TSSs, mostly based on features of PCG promoters such as CpG content, epigenetic marks, nucleosome positioning [10–19] but the results are quite mixed. A precise and complete map of miRNA TSSs/promoters is thus still missing precluding a genome-wide view of miRNA transcriptional regulations and the identification of potential miRNA-specific regulations. This lack of knowledge does not impede the study of specific miRNA loci though. We and others have shown that miRNA genes and PCGs are regulated by the same TFs. For instance, we have demonstrated that the PML-RARA oncogenic protein, which is associated with the Acute Promyelocytic Leukemia, represses the transcription of retinoic acid-responsive miRNA genes similarly to its action on PCGs [20]. Likewise, we showed that the antagonism between retinoic acid and estrogen signaling initially reported for PCGs [21] is also observed on miRNA genes [22].

At the posttranscriptional level, control of the miRNA biogenesis can be subjected to RNA-binding proteins (RBPs), which recognize specific RNA motifs on or at the vicinity of the pre-miRNAs. For instance, the LIN28 protein, a developmentally regulated RBP, can recognize a specific motif in the loop of the pre-miRNAs belonging to the let-7 family and selectively blocks their processing [23]. Also the p72 DEAD Box RNA Helicase binds a motif located in the 3′ flanking region of the pre-miRNAs and this binding can be controlled—in a cell-density-dependent manner—by the sequestration of p72 by YAP, a downstream target of the tumor-suppressive Hippo-signaling pathway [24].

These transcriptional and posttranscriptional regulations make miRNA extremely sensitive to various intra- and extracellular stimuli (e.g., hormones, vitamins, nutrients, pharmacological molecules, or hypoxia). They notably ensure that the miRNA repertoire is controlled in a temporal and cell-specific manner. These features were first reported by Chen et al., who observed that the miR-181 was preferentially expressed in the B-lymphoid cells and that its ectopic expression in hematopoietic progenitor

cells redirects lymphopoiesis towards the B-cell lineage [25]. On the other hand, these tight regulations can have severe consequences in human diseases in particular cancer [22, 26–30].

microRNA Deregulation in Cancers

The miRNAs are key players in cancer initiation and progression, including metastasis formation [31–33]. This field of research is probably one of the most productive in terms of publications (16,022 publications related to "miRNA and Cancer" listed in PubMed in March 2015 with an increase throughout the years). The miRNAs can act as oncogenes ("oncomirs") or tumor suppressors [34]. He et al. first reported the potential of one miRNA cluster, the miR-17/92, to act as an oncogene [35]. In 2007, Chang et al. showed that the miR-34a, which is transcriptionally regulated by p53, has a tumor suppressor activity [36]. Several databases have now been created to list the miRNA activity in specific cancer type [37, 38]. As observed for PCGs [39–43], the oncogene/ tumor suppressor activity of miRNAs depends on the cellular context and/or the type of cancer considered. For example, the miR-221 can act as an oncogene in liver cancer [44] while playing a tumor suppressor role in erythroblastic leukemia [45].

The miRNA deregulations observed in cancer (i.e., forced expression for oncomiRs and downregulation for tumor suppressor miRNAs) can occur at the gene (deletions, amplifications, or mutations of miRNA genes), the transcriptional (epigenetic silencing, deregulation of transcription factors), and/or the posttranscriptional (deregulation of the miRNA biogenesis pathway) levels (for review [29]). The action of miRNAs can also be impaired without affecting miRNA expression levels by, for example, genomic mutations that can modify either the sequence of the miRNAs and/or the sequence of their targets [46]. We provided earlier some examples of specific transcriptional regulations responsible for miRNA deregulations [20, 36]. Likewise, the miR-15a and miR-16 are downregulated in the majority of chronic lymphocytic leukemia cases because the corresponding gene is frequently deleted [47]. The transcription of miRNA genes can also be silenced by DNA methylation [48]. At the posttranscriptional level, the reactivation of LIN28 is many human tumors can lead to the exclusive downregulation of let-7 miRNAs [49]. The expression of several key proteins involved in the processing or the action of miRNAs (e.g., Dicer, Drosha, Argonaute 2) is perturbed in certain cancers [50, 51] with presumably broad impact on cell biology.

These deregulations ultimately generate miRNA profiles that can be associated with cancer types/subtypes and/or response to chemotherapies [52–57]. Most of these profiles have been made available in several databases. PhenomiR provides data from several studies that investigate deregulation of microRNA expression in various diseases (not only cancer) and biological processes as a systematic, manually curated resource [58]. OncomiRDB is specifically dedicated to cancers [37]. Wang et al. manually curated 2259 entries of cancer-related miRNA regulations with direct experimental evidence from approximately 9000 abstracts, covering

more than 300 miRNAs and 829 target genes across 25 cancer tissues [37]. PROGmiR is aimed at providing potential prognostic properties of miRNAs in several cancer types derived from publicly available data from Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) [59]. The next question remains to determine whether these profiles contain clinically relevant biomarkers that could serve in diagnostic, prognostic, and/or theranostic tests.

Specific Advantages of microRNAs in Cancer Diagnosis

In addition to specific mutations associated to specific cancers [46], miRNA levels can also be indicative of cancer initiation, progression, and metastasis formation. Measuring miRNA levels is relatively straightforward. Several technologies are now available to profile either a specific set of miRNAs (RT-qPCR, Nanostring, microarrays) or the whole miRNA repertoire (small RNA sequencing). Advantageously, RT-qPCR does not necessitate large amount of RNA and is highly sensitive and specific. Moreover, several assays are commercially available rendering miRNA profiling easy even in clinical practice. It is important to note that each platform has, however, its advantages and drawbacks. For instance, the use of specific RT primers [60] could be a heavy procedure compared to the universal method, which uses linkers and one common RT primer. Problems with cross-priming can also lead to specificity issues and make it difficult to distinguish miRNAs belonging to the same family and differing by 1 or 2 nucleotides only. The Nanostring technology utilizes color-coded barcodes, which hybridize with the targeted miRNAs without the need of amplification thereby providing very sensitive digital data. However, similar to microarrays, RT-qPCR and Nanostring technologies are targeted approaches that do not allow the detection of novel miRNAs that can be species- and tissue-specific [61, 62]. In that context, RNA sequencing is definitely the best way to discover novel miRNAs. It can also detect sequence variation and posttranscriptional modifications thereby providing a more complete picture of the miRNA repertoire. However, its cost is still high to be envisaged in clinics. Besides, analysis of sequencing data is still a complex process, which requires rigorous bioinformatics approaches and refined sequence algorithms.

The miRNAs can be detected in a variety of human tissue specimens, fresh or Formalin-Fixed Paraffin Embedded (FFPE), and in almost all human biological fluids (e.g., serum, plasma, saliva, urine) [63–67]. In contrast to most RNAs, circulating miRNAs are remarkably stable [68]. In fact, circulating miRNAs represent a potent mode of intercellular communication [69, 70]. The secretion of miR-105 through exosome destroys tight junctions between endothelial cells thereby facilitating metastasis propagation [70]. The molecular mechanisms responsible for the secretion of miRNAs remain largely unknown. Circulating miRNAs can be free, packed into exosomes or other microvesicles present in body fluids [71] or can be associated with (lipo)proteins (HDL [72] and Argonaute 2 protein [73]). Plethora of studies showed association between the presence of one or several extracellular circulating miRNAs in a given biological fluid and cancer initiation/progression or response to