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Gaetano Santulli *Editor*

microRNA: Basic Science

From Molecular Biology to Clinical Practice

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microRNA: Basic Science

From Molecular Biology to Clinical Practice

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Preface

This book represents one volume—focused on biology and basic science—of a trilogy exploring the functional role of microRNAs from molecular biology to clinical practice. Of the other two volumes, one addresses cancer while the other provides an ample overview on the importance of microRNA in the clinical scenario.

This volume provides a state-of-the-art outline of microRNA pathophysiology. It covers up-to-date basic notions on these tiny molecules, discussed by leading scientists in the field. An introductory chapter discussing the emerging role of microRNAs, epigenetics, and micropeptidomics opens the book, followed by a thorough description of the microRNA machinery. Then, specific aspects of these fundamental molecules are investigated at different levels: in distinctive processes (such as lipid metabolism, hematopoiesis, aging), in diverse tissues (including the cardiovascular system and endometrium), cell types (pancreatic beta cells, endothelial cells, smooth muscle cells), organelles (mitochondria), and also in the complex interaction with single proteins (as in the chapter dedicated to NF kappa B). An elegant outline summarizing the principles of microRNA target prediction alongside with the most up-to-date and effective computational approaches concludes this first volume.

As mentioned above, chapters are contributed by worldwide renowned experts, working in prestigious universities including: Harvard, Yale and Oxford; Mount Sinai School of Medicine; Ohio and Ohio State Universities; University of Texas MD Anderson Cancer Center; University of South Alabama; Cedars-Sinai Medical Center in Los Angeles; The Scripps Research Institute (La Jolla, CA); Institute for Stem Cell Research in Santa Fe Springs (CA); Laval University in Canada; Kyoto University; Akita University; Nippon Medical School; Nagoya City and Okayama University in Japan; National Neuroscience Institute in Singapore; the Hong Kong Baptist University; the HKBU Institute for Research and Continuing Education in Shenzhen, China; Institute for Communicative and Cognitive Neuroscience in Kavalappara; University of Hyderabad in India; Federation University in Australia; Hebrew University in Jerusalem; and prominent European Institutions including

Universities of Pavia, Turin, Lausanne, Montpellier, Oviedo, Aveiro, Vienna, Ljubljana and Tartu, the National University of Ireland, St. James's Hospital in Dublin, and the Royal College of Surgeons in Ireland.

Throughout these chapters, the authors spotlight forthcoming opportunities for research in basic pathophysiology and in prevention/therapy, in addition to detailed and exhaustive overviews of the current literature pertaining to microRNAs.

The book includes numerous color photographs, schemes, and diagrams of molecular pathways and tables that support and complement the text.

The comprehensive and systematic overview provided within these volumes is expected to assist the reader in comprehending the importance of taking into account the functional roles of microRNAs and also to address questions and unresolved issues regarding their importance in diagnosis and treatment of several disorders.

Finally, the editor would like to express his sincere appreciation to all the contributors for their dedicated collaboration in this project. I also wish to thank my family and the Springer team, especially Aleta, Jeff, and Diana, for their patient, professional, and constant support. I sincerely hope this book will enable readers to connect basic research principles with up-to-date clinical knowledge, thereby encouraging future discoveries and developments of new therapeutic strategies.

New York, NY, USA Gaetano Santulli, M.D., Ph.D.

The original version of the editor affiliation has been revised. An erratum can be found at DOI 10.1007/978-3-319-22380-3_13

The complexity of gene regulation by proteins alone was so enormous that I never imagined—and nobody I knew imagined—that we needed to look for new kinds of regulatory molecules.

Victor Ambros *JCB* 2013;201:492

But it is important to continue to explore the diversity of biology, and not become myopic about translating biological discovery to humans via, for example, more research on our closer relatives.

Gary Ruvkun *Nat Med* 2008;14:1041

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Chapter 1
A Fleeting Glimpse Inside microRNA, Epigenetics, and Micropeptidomics

Gaetano Santulli

Abstract MicroRNAs (miRs) are important regulators of gene expression in numerous biological processes. Their maturation process is herein described, including the most updated insights from the current literature. Circa 2000 miR sequences have been identified in the human genome, with over 50,000 miR-target interactions, including enzymes involved in epigenetic modulation of gene expression. Moreover, some "pieces of RNA" previously annotated as noncoding have been recently found to encode micropeptides that carry out critical mechanistic functions in the cell. Advanced techniques now available will certainly allow a precise scanning of the genome looking for micropeptides hidden within the "noncoding" RNA.

Keywords miRNA • Micropeptides • ORF • Micropeptidome • Mitochondria Myogenin • Humanin • SERCA • MOTS-c • Micropeptidomics • RISC • Pharmacogenomics • Drosha • Dicer • METTL3 • Exportin • TargetScan • miRWalk • miRBase • EpimiR • Transcriptome • Precision medicine

Introduction

MicroRNAs (miRs) are an evolutionarily conserved family of small (~22 nucleotides) generally [1–4] noncoding RNAs, first discovered in *Caenorhabditis elegans* [5–8]. They represent a vital component of genetic regulation, existing in virtually all organisms, suggesting thereby a pivotal role in biological processes. Undeniably, miRs are important regulators of gene expression in a plethora of biological processes including cellular proliferation, differentiation, and tumorigenesis [9–26]. Other examples of noncoding RNAs are reported in Table 1.1.

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Table 1.1 Characteristics of noncoding RNAs within the cell

Abbreviation	Complete name	Main functions	Length (nt)	Ref.
miRNA	MicroRNA	Gene silencing	21–25	[4]
rRNA	Ribosomal RNA	Translation	120–4700	[88]
tRNA	Transfer RNA	Translation	70–100	[89]
aRNA	Antisense RNA	Transcriptional attenuation	>30	[90, 91]
eRNA	Enhancer-like ncRNA (1D/2D)	Transcriptional enhancers	50–2000	[92, 93]
lincRNA	Long intergenic RNA	Transcriptional and posttranscriptional regulation	<50 kb	[94]
piRNA	PIWI-interacting RNA	Genome stabilization	24–30	[95]
shRNA	Short hairpin RNA	Gene silencing	19–29	[96]
siRNA	Short interfering RNA	Gene silencing	21–25	[97]
snRNA	Small nuclear RNA	Splicing		
snoRNA	Small nucleolar RNA	Methylation (C/D box), pseudouridylation (H/ACA box)	~20–24	[98]
SRP-RNA	Signal recognition particle RNA	Translocation of proteins across the endoplasmic reticulum		
tiRNA	Transcription initiation RNA	Transcriptional regulation	18	[99]
Y RNA	Y RNA	DNA replication and RNA processing (repressor of Ro60)	83–112	[100, 101]
CUT	Cryptic unstable transcript	Gene regulation	200–800	[102]
NAT	Natural antisense transcript	RNA interference	Variable	[103, 104]
PALR	Promoter-associated long RNA	Transcriptional regulation	200–1000	[105–107]
PROMPT	Promoter upstream transcript	Gene transcription	long	[108]
RNase P	Ribonuclease P	Endonucleolytic 5′ cleavage of tRNA precursors (ribozyme)	354–417	[109, 110]

(continued)

Table 1.1 (continued)

Abbreviation	Complete name	Main functions	Length (nt)	Ref.
RNase MRP	Mitochondrial RNA processing ribonuclease	Mitochondrial DNA replication and rRNA maturation (ribozyme)	265–340	[111, 112]
SINE	Short interspersed repetitive elements	Transcriptional suppressor (e.g. Alu element)	<500	[113, 114]
TERC	Telomerase RNA component	Telomere synthesis	451	[115, 116]
T-UCR	Transcribed ultra-conserved region	Transcriptional enhancer	>200	[117, 118]
vlincRNAs	Very long intergenic RNA	Transcriptional and posttranscriptional regulation	>50 kb	[119, 120]

Biogenesis

Classically, miRs are regarded as negative regulators of gene expression that inhibit translation and/or promote mRNA degradation by base pairing to complementary sequences within the 3′-untranslated region (3′-UTR) of protein-coding mRNA transcripts [27, 28]—mRNA degradation accounts for the majority of miR activity [29]. By altering levels of key regulators within complex genetic pathways, miRs provide a posttranscriptional level of control of homeostatic and developmental events [30–32].

Specific structural aspects of miRs are discussed in detail in Chap. 2 of this book. Briefly, maturation of miRs involves a multi-step process [33–35] that starts from the transcription (mainly operated by RNA polymerase II) of single-stranded nonprotein-coding RNAs, which are either transcribed as stand-alone transcripts (*intergenic* miRs), often encoding various miRs, or generated by the processing of introns of protein-coding genes (*intragenic* or intronic miRs). Transcription of intergenic miRs leads to the formation of primary miRs (pri-miRs) with a characteristic hairpin or stem–loop structure [36], which are subsequently processed by the nuclear RNase III, Drosha [37], and its partner proteins, including the DiGeorge Syndrome Critical Region 8 (DGCR8, known as Pasha in invertebrates), named for its association with DiGeorge Syndrome [38, 39], to become precursor miRs (pre-miRs). On the other hand, intronic miRs are obtained by the regular transcription of their host genes and then spliced to form looped pre-miRs, bypassing thereby the Drosha pathway [33, 40]. Recently, Claudio Alarcón and colleagues discovered that the addition of an m6A mark to primary miRs by methyltransferase-like 3 (METTL3) is required for their recognition by DGCR8 [41]. They also proved that METTL3 is sufficient to enhance

miR maturation in a global and non-cell-type-specific manner, acting as a strategic posttranscriptional modification that promotes the initiation of miR biogenesis.

Pre-miRs are exported from the nucleus in the cytoplasm in a process involving the Ran-GTP-dependent shuttle Exportin-5 [42]. Once in the cytosol, the pre-miR hairpin is cleaved by the RNase III enzyme Dicer [43, 44], yielding a mature miR:miR* duplex about 22 nucleotides in length, which is subsequently incorporated into the protein complex called RNA-induced silencing complex (RISC) to form miRISC [45, 46]. At this point, one of the double strands, the guide strand, is selected by the argonaute protein [47], the catalytically active RNase in the RISC complex, on the basis of the thermodynamic stability of the 5′ end. In particular, the strand with a less thermodynamically stable 5′ end is commonly chosen and loaded into the RISC complex [48], serving as a guide for mRISC to find its complementary motifs in the 3′-UTR of the target mRNA(s). Although either strand of the mature duplex may potentially act as a functional miR, only one strand is usually incorporated into the RISC where the miR and its mRNA target interact [49, 50]. Such a binding inhibits the translation of the protein that the target mRNA encodes or promotes gene silencing via mRNA degradation [51, 52].

Nearly 2000 miR sequences have been heretofore identified in the human genome, with over 50,000 miR-target interactions. Several algorithms and bioinformatics websites, including TargetScan and miRWalk [53, 54], have been developed to predict specific mRNA/miR interactions. However, miR binding rules are quite complex and not fully understood, resulting in a lack of consensus in the literature.

Given all these crucial features, miRs could represent an important way for the cell to establish intercellular (with other cells, via secreted miRs) and intracellular (among its own genes) communication. Determining direct cause-and-effect links between miRs and mRNA targets is essential to understanding the molecular mechanisms underlying disease and the subsequent development of targeted therapies [55, 56].

Walking through an Apparently Complicated Nomenclature: miR, *miR*, miR-Xa, miR*

Nomenclature of miRs may appear confusing to the naïve readers. Briefly, mature miRs are named using the non-italicized prefix “miR-” followed by a roman number (with the exception of a few early miRNAs including the let family); stem–loop precursor miRs are all named using the italicized prefix “*mir-*”.

Similar miR sequences are distinguished by a lettered suffix, for example, miR-200a, miR-200b, and miR-200c, without implying shared targets or functions. Identical miR sequences are distinguished by a numerical suffix: for instance, mir-7-1 (located on chromosome 9), mir-7-2 (located on chromosome 15), and mir-7-3 (located on chromosome 19) can all produce identical mature miRNAs. Mature miRs can be formed from either arm of the stem–loop precursor miRNA (pre-miR). In the majority of cases, one arm is more commonly formed than the other (guide strand). Previous convention was to name these strands according to their relative abundance, with the less common form (“passenger strand”) taking the name

miR-X*. However, the latest convention is to name mature miRs by the arm of the pre-miR from which they are derived, regardless of their abundance: those from the 5′ arm are named miR-X-5p and those from the 3′ arm as miR-X-3p. Therefore, miR-181a is now known as miR-181a-5p and miR-181a* is now known as miR-181a-3p, avoiding problems with the previous system if the abundance of each arm changes between tissues, developmental stages, or species. All of the above naming conventions can be preceded by a three-letter code which identifies the species the miRNA is from: hsa=*homo sapiens* (human); rno=*rattus norvegicus* (rat). Therefore, miR-181a-5p found in humans could be represented as hsa-miR-181a-5p. Of note, identical miRNAs are given the same number, regardless of species.

Epigenetics and miRs: An Intricate Affair

Epigenetics is the study of heritable changes in gene expression caused by mechanisms other than changes in the underlying DNA sequences [57], including DNA methylation [58] and posttranslational modifications of chromatin proteins [59, 60]. The main enzymes involved in this process are DNA methyltransferases (DNMT), histone demethylases (HDM), histone acetylases (HAT), and histone deacetylases (HDAC). Mounting evidence demonstrates that epigenetics and miRs can affect each other in an intricate connection [61–64]. Indeed, miRs play a key role in regulating DNA methylation or histone modifications through means of directly targeting epigenetic enzymes or functional protein complexes. For instance, a global DNA hypomethylation is induced by miR-29b leading to marked reduction of the expression of DNMT1, DNMT3A, and DNMT3B and subsequent reactivation of tumor suppressor genes p15 (INK4b) and ESR1 [65, 66]. Another example is given by miR-200a, which upregulates histone H3 acetylation via direct targeting of the 3′ untranslated region of the HDAC4 mRNA [67].

On the other hand, epigenetic control is involved in the regulation of miR expression. DNA methylation of promoter-associated CpG dinucleotides generally correlates with reduced transcription levels of corresponding miRs [68–70], thereby inducing the expression of miR target genes. A novel miR-148a/DNMT1 regulatory circuit has been identified in hepatocellular carcinogenesis: a member of the miR-148/152 family, miR-148a is a tumor suppressor that can be silenced by hypermethylation and interacts with DNMT1 [71].

A comprehensive database, EpimiR, in which experimentally validated mutual interactions between epigenetics and miRs are described, has been recently published [72].

The Emerging Functional Role of Micropeptidomics

Intriguingly, some so-called “noncoding” pieces of RNA may actually encode short proteins (micropeptides) that carry out critical mechanistic functions within the cell(s). A conserved micropeptide (46 amino acids), named myoregulin, encoded by

RNA that had been previously misannotated as noncoding, has been recently identified by Olson's group [2]. Myoregulin is a skeletal muscle-specific micropeptide that forms a transmembrane alpha helix within the membrane of the sarcoplasmic reticulum (SR), where it modulates Ca^{2+} handling interacting with the SR Ca^{2+} ATPase (SERCA).

Such a micropeptide displays a structural resemblance to phospholamban and sarcolipin, which inhibit SERCA activity in the heart and in slow-type and developing skeletal muscle [73]. The fact that putative long noncoding RNA may harbor hidden micropeptides had been suggested by recent genome-wide analyses [74]. However, heretofore the microproteome has largely been overlooked in gene annotations [75, 76].

Due to their small size, micropeptides could not be identified by genome annotation or by protein prediction algorithms whose threshold of detection is relatively high: indeed, in scans of the genome, a DNA sequence is usually not considered potentially protein-coding unless it can encode a string of more than 100 amino acids [77]. Of note, albeit some short peptides have crucial biological functions, these peptides are generally fragments chipped off larger proteins [78]. More of these "mysterious" RNA molecules could produce peptides too small to be considered true proteins but which nonetheless carry out important functions (Fig. 1.1).

Recently, other nonclassical peptides—encoded by small open reading frames (ORF)—have been discovered. These micropeptides are translated from ORF shorter than 100 amino acids. In contrast to other bioactive peptides, micropeptides are not cleaved from a larger precursor protein and lack an amino-terminal signaling sequence [79]. An estimated 40 % of mRNAs in the fruit fly *Drosophila melanogaster*, in which the first micropeptides were identified [80–82], might contain upstream ORFs in 5′-regions and some show signs of evolutionary conservation [83].

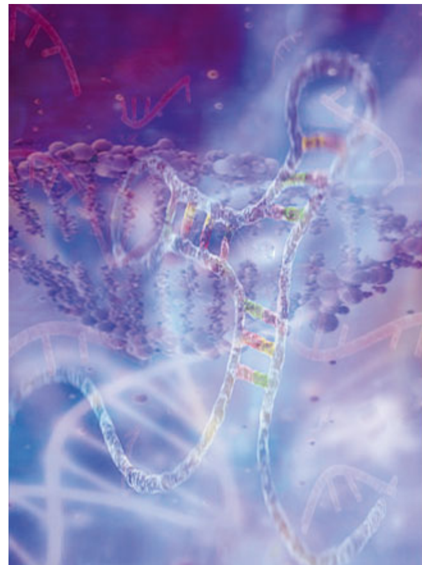

Fig. 1.1 Micropeptidomics: *multa paucis* or *hic sunt leones*?

Exploring the mitochondrial genome, a compact circular genetic system that encodes for 13 proteins essentially dedicated to energy production, Pinchas Cohen and colleagues have identified a short ORF encoded within the mitochondrial 12S rRNA that yields a bioactive peptide, named MOTS-c (mitochondrial ORF of the 12S rRNA type-c) involved in the regulation of metabolic homeostasis. The Cohen's laboratory was among the three groups [84–86] that independently discovered another important mitochondrial peptide, humanin, encoded in the mitochondrial genome by the 16S ribosomal RNA gene, MT-RNR2, which displays fundamental cytoprotective effects.

Similar short peptides could be hiding in several places in the genome, including in transcripts of unknown function. Hence, exploiting state-of-the-art techniques [1, 87], a major exciting field of research in the next years will be represented by scanning the microproteome embedded in the (previously annotated) "noncoding" RNA.

Is this the way toward precision medicine? We'll see.

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