Advances in Experimental Medicine and Biology 888

Gaetano Santulli *Editor*

microRNA: Medical Evidence

From Molecular Biology to Clinical Practice

Springer

Advances in Experimental Medicine and Biology

Volume 888

More information about this series at http://www.springer.com/series/5584

Gaetano Santulli
Editor

microRNA: Medical Evidence

From Molecular Biology to Clinical Practice

Springer

Editor
Gaetano Santulli
Columbia University Medical Center
New York Presbyterian Hospital—Manhattan
New York, NY, USA

"Federico II" University Hospital
Naples, Italy

ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-319-22670-5 ISBN 978-3-319-22671-2 (eBook)
DOI 10.1007/978-3-319-22671-2

Library of Congress Control Number: 2015955613

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Springer Science+Business Media (www.springer.com)

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

The original version of the editor affiliation has been revised. An erratum can be found at DOI 10.1007/978-3-319-22671-2_22

Contents

Contributors

Ayyappan Anitha Department of Neurogenetics, Institute for Communicative and Cognitive Neurosciences (ICCONS), Palakkad, Kerala, India

Jaideep Banerjee Extremity Trauma and Regenerative Medicine Division, US Army Institute of Surgical Research, San Antonio, TX, USA

Sébastien Bonnet Pulmonary Hypertension Research Group of the Quebec Heart and Lung Institute Research Center, Laval University, Quebec City, QC, Canada

Olivier Boucherat Pulmonary Hypertension Research Group of the University Institute of Cardiology and Pneumology Research Center, Laval University, Quebec City, QC, Canada

Fadi J. Charchar School of Applied and Biomedical Sciences, Faculty of Science and Technology, Federation University Australia, Mount Helen, VIC, Australia

Arthur C. K. Chung Partner State Key Laboratory of Environmental and Biological Analysis, and Department of Chemistry, The Hong Kong Baptist University, Hong Kong, China

HKBU Institute for Research and Continuing Education, Shenzhen, China

Petra Cimflová Department of Radiology, St. Anne's University Hospital and Faculty of Medicine, Masaryk University, Brno, Czech Republic

Dominika Coufalová Department of Anatomy, Faculty of Medicine, Masaryk University, Brno, Czech Republic

International Clinical Research Center, St. Anne's University Hospital, Brno, Czech Republic

Filipe V. Duarte Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Iris Eisenberg Department of Obstetrics and Gynecology, The Magda and Richard Hoffman Center for Human Placenta Research, Hadassah Mt. Scopus—Hebrew University Medical Center, Jerusalem, Israel

Damjan Glavač Department of Molecular Genetics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

Debra Goldman-Wohl Department of Obstetrics and Gynecology, The Magda and Richard Hoffman Center for Human Placenta Research, Hadassah Mt. Scopus—Hebrew University Medical Center, Jerusalem, Israel

David C. Henshall Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin, Ireland

Tal Imbar Department of Obstetrics and Gynecology, The Magda and Richard Hoffman Center for Human Placenta Research, Hadassah Mt. Scopus—Hebrew University Medical Center, Jerusalem, Israel

Kíra Jelencsics Medical University of Vienna, Vienna, Austria

Linda Kašičková Department of Neurology, St. Anne's University Hospital and Faculty of Medicine, Masaryk University, Brno, Czech Republic

Department of Anatomy, Faculty of Medicine, Masaryk University, Brno, Czech Republic

Noora Kotaja Department of Physiology, Institute of Biomedicine, University of Turku, Turku, Finland

Charles-Henri Lecellier University of Montpellier, Montpellier, France

Institute of Molecular Genetics of Montpellier, Montpellier, France

Computational Biology Institute, Montpellier, France

Nicolas Leuenberger Swiss Laboratory for Doping Analyses, University Center of Legal Medicine, Lausanne–Geneva, Switzerland

Lausanne University Hospital, University of Lausanne, Epalinges, Switzerland

Shi-Lung Lin Division of Regenerative Medicine, WJWU & LYNN Institute for Stem Cell Research, Santa Fe Springs, CA, USA

Jun Lu Department of Genetics, Yale Stem Cell Center and Yale Cancer Center, Yale University School of Medicine, New Haven, CT, USA

Yale Center for RNA Science and Medicine, New Haven, CT, USA

Francine Z. Marques School of Applied and Biomedical Sciences, Faculty of Science and Technology, Federation University Australia, Mount Helen, VIC, Australia

Heart Failure Research Group, Baker IDI Heart and Diabetes Institute, Melbourne, VIC, Australia

Stephen Mastriano Department of Genetics, Yale Stem Cell Center and Yale Cancer Center, Yale University School of Medicine, New Haven, CT, USA

Yutaka Naito Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo, Japan

Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Jan Novák 2nd Department of Internal Medicine, St. Anne's University Hospital and Faculty of Medicine, Masaryk University, Brno, Czech Republic

Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic

Department of Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic

Rainer Oberbauer Medical University of Vienna, Vienna, Austria

Takahiro Ochiya Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo, Japan

Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Koh Ono Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan

Carlos M. Palmeira Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Department of Life Sciences, University of Coimbra, Coimbra, Portugal

François Potus Pulmonary Hypertension Research Group of the Quebec Heart and Lung Institute Research Center, Laval University, Quebec City, QC, Canada

Lifeng Qiu Neural Stem Cell Research Lab, Department of Research, National Neuroscience Institute, Singapore, Singapore

Metka Ravnik-Glavač Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

Ana Rebane Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia

Cristina R. Reschke Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin 2, Ireland

Christine Roden Department of Genetics, Yale Stem Cell Center and Yale Cancer Center, Yale University School of Medicine, New Haven, CT, USA

Anabela P. Rolo Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Department of Life Sciences, University of Coimbra, Coimbra, Portugal

Atsushi Sakai Department of Pharmacology, Graduate School of Medicine, Nippon Medical School, Tokyo, Japan

Gaetano Santulli Columbia University Medical Center, New York Presbyterian Hospital—Manhattan, New York, NY, USA

"Federico II" University Hospital, Naples, Italy

Martial Saugy Swiss Laboratory for Doping Analyses, University Center of Legal Medicine, Lausanne–Geneva, Switzerland

Lausanne University Hospital and University of Lausanne, Epalinges, Switzerland

Anne Saumet University of Montpelier, Montpellier, France

Faculty of Medicine, Montpellier, France

Chandan K. Sen The Ohio State University Wexner Medical Center, Davis Heart & Lung Research Institute, Comprehensive Wound Center, Center for Regenerative Medicine & Cell-Based Therapies, Columbus, OH, USA

Hidenori Suzuki Department of Pharmacology, Graduate School of Medicine, Nippon Medical School, Tokyo, Japan

Eng King Tan Department of Neurology, National Neuroscience Institute, Singapore, Singapore

Department of Research, National Neuroscience Institute, Singapore, Singapore

Neuroscience and Behavioral Disorders program, Duke-National University of Singapore Graduate Medical School, Singapore, Singapore

Yasuhito Tanaka Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo, Japan

Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Ismail Thanseem Department of Neurogenetics, Institute for Communicative and Cognitive Neurosciences (ICCONS), Palakkad, Kerala, India

Ondřej Volný Department of Neurology, St. Anne's University Hospital and Faculty of Medicine, Masaryk University, Brno, Czech Republic

Department of Anatomy, Faculty of Medicine, Masaryk University, Brno, Czech Republic

International Clinical Research Center, St. Anne's University Hospital, Brno, Czech Republic

Nayi Wang The Biomedical Engineering Graduate Program, New Haven, CT, USA

Li Zeng Neural Stem Cell Research Lab, Department of Research, National Neuroscience Institute, Singapore, Singapore

Department of Research, National Neuroscience Institute, Singapore, Singapore

Neuroscience and Behavioral Disorders program, Duke-National University of Singapore Graduate Medical School, Singapore, Singapore

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].

The original version of this chapter was revised. The erratum to this chapter is available at: DOI 10.1007/978-3-319-22671-2_22

G. Santulli, M.D., Ph.D. (✉)
Columbia University Medical Center, New York Presbyterian Hospital—Manhattan, New York, NY, USA

"Federico II" University Hospital, Naples, Italy
e-mail: gsantulli001@gmail.com

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_1

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.

Acknowledgements Dr. Santulli is supported by the NIH (1K99DK107895).

References

1. McCarthy M. Obama promises to defend health law and promote "precision medicine". BMJ. 2015;350:h385.
2. Elwood TW. The usefulness of discerning patterns. J Allied Health. 2015;44:1.
3. Collins FS, Varmus H. A new initiative on precision medicine. N Engl J Med. 2015;372:793–5.
4. Fox JL. Obama catapults patient-empowered Precision Medicine. Nat Biotechnol. 2015;33:325.
5. Lin AH, Timberlake N, Logg CR, Liu Y, Kamijima S, Diago O, et al. MicroRNA 142-3p attenuates spread of replicating retroviral vector in hematopoietic lineage-derived cells while maintaining an antiviral immune response. Hum Gene Ther. 2014;25:759–71.
6. Santulli G, Wronska A, Uryu K, Diacovo TG, Gao M, Marx SO, et al. A selective microRNA-based strategy inhibits restenosis while preserving endothelial function. J Clin Invest. 2014;124:4102–14.

7. Edwards JK, Pasqualini R, Arap W, Calin GA. MicroRNAs and ultraconserved genes as diagnostic markers and therapeutic targets in cancer and cardiovascular diseases. J Cardiovasc Transl Res. 2010;3:271–9.
8. Ristori E, Lopez-Ramirez MA, Narayanan A, Hill-Teran G, Moro A, Calvo CF, et al. A dicer-miR-107 interaction regulates biogenesis of specific miRNAs crucial for neurogenesis. Dev Cell. 2015;32:546–60.
9. Santulli G, Iaccarino G, De Luca N, Trimarco B, Condorelli G. Atrial fibrillation and microRNAs. Front Physiol. 2014;5:15.
10. Della Peruta M, Badar A, Rosales C, Chokshi S, Kia A, Nathwani D, et al. Preferential targeting of disseminated liver tumors using a recombinant adeno-associated viral vector. Hum Gene Ther. 2015;26:94–103.
11. Sardu C, Marfella R, Santulli G, Paolisso G. Functional role of miRNA in cardiac resynchronization therapy. Pharmacogenomics. 2014;15:1159–68.
12. Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, et al. A brain-specific microRNA regulates dendritic spine development. Nature. 2006;439:283–9.
13. Taulli R, Pandolfi PP. "Snorkeling" for missing players in cancer. J Clin Invest. 2012;122:2765–8.
14. Wronska A, Kurkowska-Jastrzebska I, Santulli G. Application of microRNAs in diagnosis and treatment of cardiovascular disease. Acta Physiol (Oxf). 2015;213:60–83.

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.

Keywords Cancer • Theranosis • Diagnosis • Prognosis • Precision medicine • Tumorigenesis

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

A. Saumet, M.Sc., Ph.D.
University of Montpellier, 163 rue Auguste Broussonnet, Montpellier 34090, France

Faculty of Medicine, 2 rue de l'Ecole de Médecine, Montpellier 34060, France
e-mail: annesaumet@yahoo.fr

C.-H. Lecellier, M.Sc., Ph.D. (✉)
University of Montpellier, 163 rue Auguste Broussonnet, Montpellier 34090, France

Institute of Molecular Genetics of Montpellier, CNRS UMR 5535,
1919 Route de Mende, Montpellier 34293, France

Computational Biology Institute, 860 rue de Saint Priest, Montpellier 34095, France
e-mail: charles.lecellier@igmm.cnrs.fr

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_2

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

chemotherapy. These profiles have been listed and classified in the miRandola database [74]. miRandola contains 2132 entries, with 581 unique mature miRNAs and 21 types of samples. miRNAs are classified into four categories, based on their extracellular form: miRNA-Ago2 (173 entries), miRNA-exosome (856 entries), miRNA-HDL (20 entries), and miRNA-circulating (1083 entries) [74]. miRandola is also connected to miRò, a compendium, which integrates various online resources (ontologies, diseases, and targets) to provide users with miRNA-phenotype associations in humans [75].

All these features make miRNAs appealing candidates for non-invasive diagnostic tests and several companies have indeed decided to meet the challenge (e.g., Santaris Pharma, Rosetta Genomics, Cepheid, Prestizia-Theradiag, and IntegraGen; [76]). However, at this stage, miRNA signatures per cancer type are still inconsistent [77, 78] impeding their usage in clinics and calling for further development and research.

Challenges in microRNA-Based Diagnosis

One important challenge in the field of microRNA-based diagnosis is to find the sources of inconsistencies in order to propose standardized protocols. Inconsistencies in miRNA signatures could come from sample procurement and could be the results of, for instance, platelet contamination of the plasma [79, 80] or hemolysis occurring during blood collection [81–84]. The protocols used to extract miRNAs also differ and can introduce significant variability. One important point to compare miRNA extraction protocols is to evaluate the quantity and the quality of the extracted miRNAs. Though the size and abundance of ribosomal RNAs is traditionally used as a quality marker for large RNAs, these RNAs cannot be informative on the quality of the miRNA extraction and specific methods are required (e.g., Agilent Small RNA Kit, synthetic miRNA standards). Moreover the quantification of miRNAs is only accurate in samples where larger RNAs are not degraded. The low concentration of RNAs in body fluids also makes the estimation of miRNAs abundance particularly difficult [85]. Besides, protein and lipid content of plasma and serum samples could affect efficiency of RNA extraction and introduce potential inhibitors of PCR [86]. This can be estimated using a spiked non-human synthetic miRNAs (typically from Arabidopsis thaliana or *Caenorhabditis elegans*) that will go through the entire RNA isolation procedure and will eventually be measured by RT-qPCR. Another aspect that should be considered is that the extraction methods could affect the nature (i.e., nucleotide composition) of the miRNAs extracted. Notably, depending on the protocol used, the quantity of the biological samples can impact the GC content of the miRNAs detected [87, 88]. Since these observations were also made in serum [88] (where large RNAs are barely detected), it is likely that the selective lost of miRNAs is linked to the presence of additional compounds (proteins and/or lipids), which are associated with miRNAs. Together these studies [87, 88] argue for standardization in quantities/volumes of starting materials to allow strict comparison of miRNA profiles. In fact, all these considerations point to the urgent need of consistency in all the steps of miRNA extraction procedures.

Analytical aspects also impact the definition of miRNA signatures. Among them, normalization of the data, which is required to remove unwanted technical variation present in the samples, is critical. On common approach is to use other abundant noncoding RNAs, such as U6 small nuclear RNAs, as normalizers of miRNA expression. However, the biology of such RNAs is quite distinct from miRNA biology in terms of transcription, processing, and tissue-specific expression [89]. An alternative is to use miRNAs whose expression is supposed to be stable in various conditions. However, this strategy can be limited by the fact that the chosen reference miRNAs are sensitive to other biological processes and/or other diseases commonly encountered in clinics. In that case, the expression of the normalizer miRNAs could fluctuate in patients and introduce serious bias. In fact miRNA levels are extremely sensitive to various stimuli and conditions, even nonpathogenic, from gender [90, 91] and age [92, 93] to nutrients such as amino acids, carbohydrates, fatty acids, vitamins, and phytochemicals (curcumin, resveratrol) [94, 95]. If clinically relevant, these aspects should be invariably taken into account in the cohort used to define a miRNA signature. The ideal strategy would be to restrict potential miRNA signature to miRNAs whose transcriptional/posttranscriptional regulations are relevant for the cancer or the chemotherapy considered. This is where translational research meets fundamental research as this strategy clearly depends on a better understanding of miRNA regulations.

Conclusion

The discovery of miRNAs [96] has opened up new avenues of research in biomedicine, in particular in cancer, and contributed to a large extent to the "Noncoding RNA revolution" [97]. It is remarkable to note not only the fast rate of fundamental discoveries made in two decades (illustrated by the exponentially growing number of publications) but also the velocity with which "miRNA gets to business" [76]. These molecules indeed harbor specific features (stability, easy manipulation, reasonably simple detection, tissue specificity) that make them appealing candidates as diagnostic, prognostic, or theranostic biomarkers and even therapeutic targets [64, 98]. However some uncertainties remain [77, 78] that may prevent their immediate large-scale exploitation. Collective efforts made by clinicians, academic and industrial researchers are needed to circumvent these limitations and promote the transfer of miRNAs from bench to bedside.

References

1. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol. 2009;10:126–39.
2. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet. 2010;11:597–610.

3. Tran N, Hutvagner G. Biogenesis and the regulation of the maturation of miRNAs. Essays Biochem. 2013;54:17–28.
4. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. Processing of primary microRNAs by the microprocessor complex. Nature. 2004;432:231–5.
5. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN. The nuclear RNase III Drosha initiates microRNA processing. Nature. 2003;425:415–9.
6. Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev. 2004;18:3016–27.
7. Ha M, Kim VN. Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol. 2014;15:509–24.
8. Huntzinger E, Izaurralde E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet. 2011;12:99–110.
9. Conrad T, Marsico A, Gehre M, Orom UA. Microprocessor activity controls differential miRNA biogenesis In Vivo. Cell Rep. 2014;9:542–54.
10. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M, Lin C, Socci ND, Hermida L, Fulci V, Chiaretti S, Foa R, Schliwka J, Fuchs U, Novosel A, Muller RU, Schermer B, Bissels U, Inman J, Phan Q, Chien M, Weir DB, Choksi R, De Vita G, Frezzetti D, Trompeter HI, Hornung V, Teng G, Hartmann G, Palkovits M, Di Lauro R, Wernet P, Macino G, Rogler CE, Nagle JW, Ju J, Papavasiliou FN, Benzing T, Lichter P, Tam W, Brownstein MJ, Bosio A, Borkhardt A, Russo JJ, Sander C, Zavolan M, Tuschl T. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell. 2007;129:1401–14.
11. Saini HK, Griffiths-Jones S, Enright AJ. Genomic analysis of human microRNA transcripts. Proc Natl Acad Sci U S A. 2007;104:17719–24.
12. Fujita S, Iba H. Putative promoter regions of miRNA genes involved in evolutionarily conserved regulatory systems among vertebrates. Bioinformatics. 2008;24:303–8.
13. Marsico A, Huska MR, Lasserre J, Hu H, Vucicevic D, Musahl A, Orom U, Vingron M. PROmiRNA: a new miRNA promoter recognition method uncovers the complex regulation of intronic miRNAs. Genome Biol. 2013;14:R84.
14. Ozsolak F, Poling LL, Wang Z, Liu H, Liu XS, Roeder RG, Zhang X, Song JS, Fisher DE. Chromatin structure analyses identify miRNA promoters. Genes Dev. 2008;22:3172–83.
15. Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, Guenther MG, Johnston WK, Wernig M, Newman J, Calabrese JM, Dennis LM, Volkert TL, Gupta S, Love J, Hannett N, Sharp PA, Bartel DP, Jaenisch R, Young RA. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell. 2008;134:521–33.
16. Corcoran DL, Pandit KV, Gordon B, Bhattacharjee A, Kaminski N, Benos PV. Features of mammalian microRNA promoters emerge from polymerase II chromatin immunoprecipitation data. PLoS One. 2009;4, e5279.
17. Chien CH, Sun YM, Chang WC, Chiang-Hsieh PY, Lee TY, Tsai WC, Horng JT, Tsou AP, Huang HD. Identifying transcriptional start sites of human microRNAs based on high-throughput sequencing data. Nucleic Acids Res. 2011;39:9345–56.
18. Chen D, Fu LY, Zhang Z, Li G, Zhang H, Jiang L, Harrison AP, Shanahan HP, Klukas C, Zhang HY, Ruan Y, Chen LL, Chen M. Dissecting the chromatin interactome of microRNA genes. Nucleic Acids Res. 2014;42:3028–43.
19. Georgakilas G, Vlachos IS, Paraskevopoulou MD, Yang P, Zhang Y, Economides AN, Hatzigeorgiou AG. microTSS: accurate microRNA transcription start site identification reveals a significant number of divergent pri-miRNAs. Nat Commun. 2014;5:5700.
20. Saumet A, Vetter G, Bouttier M, Portales-Casamar E, Wasserman WW, Maurin T, Mari B, Barbry P, Vallar L, Friederich E, Arar K, Cassinat B, Chomienne C, Lecellier CH. Transcriptional repression of microRNA genes by PML-RARA increases expression of key cancer proteins in acute promyelocytic leukemia. Blood. 2009;113:412–21.
21. Hua S, Kittler R, White KP. Genomic antagonism between retinoic acid and estrogen signaling in breast cancer. Cell. 2009;137:1259–71.
22. Saumet A, Vetter G, Bouttier M, Antoine E, Roubert C, Orsetti B, Theillet C, Lecellier CH. Estrogen and retinoic acid antagonistically regulate several microRNA genes to control aerobic glycolysis in breast cancer cells. Mol Biosyst. 2012;8:3242–53.

23. Viswanathan SR, Daley GQ, Gregory RI. Selective blockade of microRNA processing by Lin28. Science. 2008;320:97–100.
24. Mori M, Triboulet R, Mohseni M, Schlegelmilch K, Shrestha K, Camargo FD, Gregory RI. Hippo signaling regulates microprocessor and links cell-density-dependent miRNA biogenesis to cancer. Cell. 2014;156:893–906.
25. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. Science. 2004;303:83–6.
26. Waltering KK, Porkka KP, Jalava SE, Urbanucci A, Kohonen PJ, Latonen LM, Kallioniemi OP, Jenster G, Visakorpi T. Androgen regulation of micro-RNAs in prostate cancer. Prostate. 2011;71:604–14.
27. Wang WL, Chatterjee N, Chittur SV, Welsh J, Tenniswood MP. Effects of 1alpha,25 dihydroxyvitamin D3 and testosterone on miRNA and mRNA expression in LNCaP cells. Mol Cancer. 2011;10:58.
28. Kulshreshtha R, Ferracin M, Wojcik SE, Garzon R, Alder H, Agosto-Perez FJ, Davuluri R, Liu CG, Croce CM, Negrini M, Calin GA, Ivan M. A microRNA signature of hypoxia. Mol Cell Biol. 2007;27:1859–67.
29. Iorio MV, Croce CM. Causes and consequences of microRNA dysregulation. Cancer J. 2012;18:215–22.
30. Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet. 2009;10:704–14.
31. Pencheva N, Tavazoie SF. Control of metastatic progression by microRNA regulatory networks. Nat Cell Biol. 2013;15:546–54.
32. Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, Patrawala L, Yan H, Jeter C, Honorio S, Wiggins JF, Bader AG, Fagin R, Brown D, Tang DG. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. Nat Med. 2011;17:211–5.
33. Huang Q, Gumireddy K, Schrier M, le Sage C, Nagel R, Nair S, Egan DA, Li A, Huang G, Klein-Szanto AJ, Gimotty PA, Katsaros D, Coukos G, Zhang L, Pure E, Agami R. The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis. Nat Cell Biol. 2008;10:202–10.
34. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006;6:857–66.
35. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, Hammond SM. A microRNA polycistron as a potential human oncogene. Nature. 2005;435:828–33.
36. Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, Feldmann G, Yamakuchi M, Ferlito M, Lowenstein CJ, Arking DE, Beer MA, Maitra A, Mendell JT. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell. 2007;26:745–52.
37. Wang D, Gu J, Wang T, Ding Z. OncomiRDB: a database for the experimentally verified oncogenic and tumor-suppressive microRNAs. Bioinformatics. 2014;30:2237–8.
38. Li Y, Qiu C, Tu J, Geng B, Yang J, Jiang T, Cui Q. HMDD v2.0: a database for experimentally supported human microRNA and disease associations. Nucleic Acids Res. 2014;42:D1070–4.
39. Johnson DG. The paradox of E2F1: oncogene and tumor suppressor gene. Mol Carcinog. 2000;27:151–7.
40. Rowland BD, Bernards R, Peeper DS. The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. Nat Cell Biol. 2005;7:1074–82.
41. Liang J, Mills GB. AMPK: a contextual oncogene or tumor suppressor? Cancer Res. 2013;73:2929–35.
42. Toker A, Chin YR. Akt-ing up on SRPK1: oncogene or tumor suppressor? Mol Cell. 2014;54:329–30.
43. Lobry C, Oh P, Mansour MR, Look AT, Aifantis I. Notch signaling: switching an oncogene to a tumor suppressor. Blood. 2014;123:2451–9.
44. Pineau P, Volinia S, McJunkin K, Marchio A, Battiston C, Terris B, Mazzaferro V, Lowe SW, Croce CM, Dejean A. miR-221 overexpression contributes to liver tumorigenesis. Proc Natl Acad Sci U S A. 2010;107:264–9.

45. Felli N, Fontana L, Pelosi E, Botta R, Bonci D, Facchiano F, Liuzzi F, Lulli V, Morsilli O, Santoro S, Valtieri M, Calin GA, Liu CG, Sorrentino A, Croce CM, Peschle C. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. Proc Natl Acad Sci U S A. 2005;102:18081–6.
46. Mishra PJ, Banerjee D, Bertino JR. MiRSNPs or MiR-polymorphisms, new players in microRNA mediated regulation of the cell: Introducing microRNA pharmacogenomics. Cell Cycle. 2008;7:853–8.
47. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2002;99:15524–9.
48. Lopez-Serra P, Esteller M. DNA methylation-associated silencing of tumor-suppressor microRNAs in cancer. Oncogene. 2012;31:1609–22.
49. Thornton JE, Gregory RI. How does Lin28 let-7 control development and disease? Trends Cell Biol. 2012;22:474–82.
50. Blenkiron C, Goldstein LD, Thorne NP, Spiteri I, Chin SF, Dunning MJ, Barbosa-Morais NL, Teschendorff AE, Green AR, Ellis IO, Tavare S, Caldas C, Miska EA. MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biol. 2007;8:R214.
51. Avery-Kiejda KA, Braye SG, Forbes JF, Scott RJ. The expression of Dicer and Drosha in matched normal tissues, tumours and lymph node metastases in triple negative breast cancer. BMC Cancer. 2014;14:253.
52. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006;103:2257–61.
53. Di Leva G, Croce CM. miRNA profiling of cancer. Curr Opin Genet Dev. 2013;23:3–11.
54. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Menard S, Palazzo JP, Rosenberg A, Musiani P, Volinia S, Nenci I, Calin GA, Querzoli P, Negrini M, Croce CM. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 2005;65:7065–70.
55. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. Nature. 2005;435:834–8.
56. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, Iorio MV, Visone R, Sever NI, Fabbri M, Iuliano R, Palumbo T, Pichiorri F, Roldo C, Garzon R, Sevignani C, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med. 2005;353:1793–801.
57. Lowery AJ, Miller N, Devaney A, McNeill RE, Davoren PA, Lemetre C, Benes V, Schmidt S, Blake J, Ball G, Kerin MJ. MicroRNA signatures predict oestrogen receptor, progesterone receptor and HER2/neu receptor status in breast cancer. Breast Cancer Res. 2009;11:R27.
58. Ruepp A, Kowarsch A, Schmidl D, Bruggenthin F, Brauner B, Dunger I, Fobo G, Frishman G, Montrone C, Theis FJ. PhenomiR: a knowledgebase for microRNA expression in diseases and biological processes. Genome Biol. 2010;11:R6.
59. Goswami CP, Nakshatri H. PROGmiR: a tool for identifying prognostic miRNA biomarkers in multiple cancers using publicly available data. J Clin Bioinforma. 2012;2:23.
60. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005;33, e179.
61. Friedlander MR, Lizano E, Houben AJ, Bezdan D, Banez-Coronel M, Kudla G, Mateu-Huertas E, Kagerbauer B, Gonzalez J, Chen KC, LeProust EM, Marti E, Estivill X. Evidence for the biogenesis of more than 1,000 novel human microRNAs. Genome Biol. 2014;15:R57.
62. Londin E, Loher P, Telonis AG, Quann K, Clark P, Jing Y, Hatzimichael E, Kirino Y, Honda S, Lally M, Ramratnam B, Comstock CE, Knudsen KE, Gomella L, Spaeth GL, Hark L, Katz LJ,

Witkiewicz A, Rostami A, Jimenez SA, Hollingsworth MA, Yeh JJ, Shaw CA, McKenzie SE, Bray P, Nelson PT, Zupo S, Van Roosbroeck K, Keating MJ, Calin GA, Yeo C, Jimbo M, Cozzitorto J, Brody JR, Delgrosso K, Mattick JS, Fortina P, Rigoutsos I. Analysis of 13 cell types reveals evidence for the expression of numerous novel primate- and tissue-specific microRNAs. Proc Natl Acad Sci U S A. 2015;112:E1106–15.
63. Etheridge A, Lee I, Hood L, Galas D, Wang K. Extracellular microRNA: a new source of biomarkers. Mutat Res. 2011;717:85–90.
64. Heneghan HM, Miller N, Kerin MJ. MiRNAs as biomarkers and therapeutic targets in cancer. Curr Opin Pharmacol. 2010;10:543–50.
65. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105:10513–8.
66. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Zen K, Zhang CY. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008;18:997–1006.
67. Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, Calin GA. MicroRNAs in body fluids--the mix of hormones and biomarkers. Nat Rev Clin Oncol. 2011;8:467–77.
68. Chevillet JR, Lee I, Briggs HA, He Y, Wang K. Issues and prospects of microRNA-based biomarkers in blood and other body fluids. Molecules. 2014;19:6080–105.
69. Fabbri M, Paone A, Calore F, Galli R, Gaudio E, Santhanam R, Lovat F, Fadda P, Mao C, Nuovo GJ, Zanesi N, Crawford M, Ozer GH, Wernicke D, Alder H, Caligiuri MA, Nana-Sinkam P, Perrotti D, Croce CM. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. Proc Natl Acad Sci U S A. 2012;109:E2110–6.
70. Zhou W, Fong MY, Min Y, Somlo G, Liu L, Palomares MR, Yu Y, Chow A, O'Connor ST, Chin AR, Yen Y, Wang Y, Marcusson EG, Chu P, Wu J, Wu X, Li AX, Li Z, Gao H, Ren X, Boldin MP, Lin PC, Wang SE. Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. Cancer Cell. 2014;25:501–15.
71. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9:654–9.
72. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol. 2011;13:423–33.
73. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A. 2011;108:5003–8.
74. Russo F, Di Bella S, Nigita G, Macca V, Lagana A, Giugno R, Pulvirenti A, Ferro A. miRandola: extracellular circulating microRNAs database. PLoS One. 2012;7, e47786.
75. Lagana A, Forte S, Giudice A, Arena MR, Puglisi PL, Giugno R, Pulvirenti A, Shasha D, Ferro A. miRo: a miRNA knowledge base. Database (Oxford). 2009;2009:bap008.
76. Mack GS. MicroRNA gets down to business. Nat Biotechnol. 2007;25:631–8.
77. Leidner RS, Li L, Thompson CL. Dampening enthusiasm for circulating microRNA in breast cancer. PLoS One. 2013;8, e57841.
78. Jarry J, Schadendorf D, Greenwood C, Spatz A, van Kempen LC. The validity of circulating microRNAs in oncology: five years of challenges and contradictions. Mol Oncol. 2014;8:819–29.
79. Cheng HH, Yi HS, Kim Y, Kroh EM, Chien JW, Eaton KD, Goodman MT, Tait JF, Tewari M, Pritchard CC. Plasma processing conditions substantially influence circulating microRNA biomarker levels. PLoS One. 2013;8, e64795.
80. Wang K, Yuan Y, Cho JH, McClarty S, Baxter D, Galas DJ. Comparing the MicroRNA spectrum between serum and plasma. PLoS One. 2012;7, e41561.

81. Kirschner MB, Edelman JJ, Kao SC, Vallely MP, van Zandwijk N, Reid G. The impact of hemolysis on cell-free microRNA biomarkers. Front Genet. 2013;4:94.
82. Kirschner MB, Kao SC, Edelman JJ, Armstrong NJ, Vallely MP, van Zandwijk N, Reid G. Haemolysis during sample preparation alters microRNA content of plasma. PLoS One. 2011;6, e24145.
83. Blondal T, Jensby Nielsen S, Baker A, Andreasen D, Mouritzen P, Wrang Teilum M, Dahlsveen IK. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. Methods. 2013;59:S1–6.
84. Pritchard CC, Kroh E, Wood B, Arroyo JD, Dougherty KJ, Miyaji MM, Tait JF, Tewari M. Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. Cancer Prev Res (Phila). 2012;5:492–7.
85. Tzimagiorgis G, Michailidou EZ, Kritis A, Markopoulos AK, Kouidou S. Recovering circulating extracellular or cell-free RNA from bodily fluids. Cancer Epidemiol. 2011;35:580–9.
86. Kroh EM, Parkin RK, Mitchell PS, Tewari M. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). Methods. 2010;50:298–301.
87. Kim YK, Yeo J, Kim B, Ha M, Kim VN. Short structured RNAs with low GC content are selectively lost during extraction from a small number of cells. Mol Cell. 2012;46:893–5.
88. Monleau M, Bonnel S, Gostan T, Blanchard D, Courgnaud V, Lecellier CH. Comparison of different extraction techniques to profile microRNAs from human sera and peripheral blood mononuclear cells. BMC Genomics. 2014;15:395.
89. Chugh P, Dittmer DP. Potential pitfalls in microRNA profiling. Wiley Interdiscip Rev RNA. 2012;3:601–16.
90. Morgan CP, Bale TL. Sex differences in microRNA regulation of gene expression: no smoke, just miRs. Biol Sex Differ. 2012;3:22.
91. Sharma S, Eghbali M. Influence of sex differences on microRNA gene regulation in disease. Biol Sex Differ. 2014;5:3.
92. Lai CY, Wu YT, Yu SL, Yu YH, Lee SY, Liu CM, Hsieh WS, Hwu HG, Chen PC, Jeng SF, Chen WJ. Modulated expression of human peripheral blood microRNAs from infancy to adulthood and its role in aging. Aging Cell. 2014;13:679–89.
93. Noren Hooten N, Fitzpatrick M, Wood 3rd WH, De S, Ejiogu N, Zhang Y, Mattison JA, Becker KG, Zonderman AB, Evans MK. Age-related changes in microRNA levels in serum. Aging (Albany NY). 2013;5:725–40.
94. Palmer JD, Soule BP, Simone BA, Zaorsky NG, Jin L, Simone NL. MicroRNA expression altered by diet: can food be medicinal? Ageing Res Rev. 2014;17:16–24.
95. Garcia-Segura L, Perez-Andrade M, Miranda-Rios J. The emerging role of MicroRNAs in the regulation of gene expression by nutrients. J Nutrigenet Nutrigenomics. 2013;6:16–31.
96. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75:843–54.
97. Cech TR, Steitz JA. The noncoding RNA revolution-trashing old rules to forge new ones. Cell. 2014;157:77–94.
98. Hauptman N, Glavac D. MicroRNAs and long non-coding RNAs: prospects in diagnostics and therapy of cancer. Radiol Oncol. 2013;47:311–8.

Chapter 3
microRNA and Pain

Atsushi Sakai and Hidenori Suzuki

Abstract Pain is an important protective system that alerts organisms to actual or possible tissue damage. However, a variety of pathologies can lead to chronic pain that is no longer beneficial. Lesions or diseases of the somatosensory nervous system cause intractable neuropathic pain that occasionally lasts even after the original pathology subsides. Chronic inflammatory diseases like arthritis are also associated with severe pain. Because conventional analgesics such as non-steroidal anti-inflammatory drugs and opioids have limited efficacy and/or severe adverse events associated with long-term use, chronic pain remains a major problem in clinical practice. Recently, causal roles of microRNAs in chronic pain and their therapeutic potential have been emerging. microRNA expressions are altered not only at the primary origin of pain, but also along the somatosensory pathways. Notably, microRNA expressions are differentially affected depending on the causes of chronic pain. This chapter summarizes current insights into the roles of microRNAs in pain based on the underlying pathologies.

Keywords Arthritis • Cancer pain • Inflammatory pain • Neuropathic pain • Somatosensory pathways

Introduction

In general, pain is elicited by nociceptive stimuli applied to the body or through pathology in an internal organ, and is perceived in the brain [1]. A subset of primary afferents, or nociceptors, detect various forms of nociceptive stimuli, including mechanical, thermal (hot or cold), and chemical stimuli, in the body surface (skin), deep tissue, viscera, and others. Primary afferents are the axons of primary sensory neurons referred to as dorsal root ganglion (DRG) or trigeminal ganglion (TG) neurons after the locations of their cell bodies. Thus, a subset of DRG and TG neurons are the first-line nociceptive neurons that detect noxious stimuli and tissue lesions,

A. Sakai • H. Suzuki (✉)
Department of Pharmacology, Graduate School of Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan
e-mail: sa19@nms.ac.jp; hsuzuki@nms.ac.jp

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_3

while another subset of DRG neurons detect non-noxious stimuli such as tactile stimuli. Once stimulated, DRG and TG neurons transduce sensory stimuli into electrical signals and transmit these signals to the spinal and medullary dorsal horns, respectively. The sensory information is then synaptically transmitted to spinal neurons and considerably modulated by a complex spinal network interconnected with excitatory and inhibitory interneurons, descending axons from the brainstem and glial cells (microglia and astrocytes) [2, 3]. The processed sensory information is further transmitted through direct or indirect connections to multiple brain areas, including not only somatosensory cortices, but also limbic systems and other cortices. The somatosensory cortices are mainly involved in the sensory aspects of pain such as intensity and location, while the limbic systems are involved in the affective and emotional aspects of pain as well as pain perception and attention [4].

Although pain is an essential protective system that alerts organisms to actual or possible tissue damage, unnecessary or long-lasting pain is debilitating and requires medical intervention. In particular, chronic pain is a major clinical problem because conventional analgesics such as nonsteroidal anti-inflammatory drugs and opioids have problems associated with long-term treatment. In addition, neuropathic pain, a form of chronic pain caused by lesions or diseases of the somatosensory system, is no longer beneficial, and is poorly controlled by the currently available analgesics [5]. However, there are many obstacles for the development of ideal analgesics. Processing of nociceptive information is readily disrupted in injured or pathological conditions through neural plasticity that develops at multiple points along the sensory circuits [1, 6]. These changes in nociceptive processing can occur over a wide range of time scales (acute to chronic) and at multiple levels in molecules, synapses, cells, and networks [2]. For example, hyperexcitability of primary sensory neurons is commonly observed in pathological pain and is caused by changes in the expression level, intracellular distribution, and posttranslational modulation of ion channels, such as voltage-gated sodium channels [7]. In the spinal dorsal horn, aberrant processing of sensory inputs occurs through neuronal or synaptic changes such as long-term potentiation and disinhibition and contributes to pathological pain with both spinal and peripheral origins [8]. Consistent with these long-term changes in the nociceptive pathway, there is increasing evidence that epigenetic mechanisms such as DNA methylation, histone modification, and miRNA expressions are implicated in chronic pain syndromes [9]. On the other hand, spinal glial cells, especially microglia and astrocytes, are also recognized as major players in pain modulation by regulating neurotransmission and neuroinflammation [10]. In the brain, nociceptive inputs negatively affect emotion, cognition, and motivation, most notably in the chronic pain state, and chronic pain is correlated with comorbid cognitive, mood and anxiety disorders [11]. Reciprocally, the cortical activity underlying these higher brain functions can affect pain perception [12, 13].

Critical roles of microRNAs (miRNAs) have been emerging in the development and pathophysiology of the nervous system [14–16], including chronic pain [17]. miRNA dysregulation leads to abnormal neuronal excitability through regulation of ion channel expressions [18]. Specific deletion of Dicer in nociceptive DRG neurons resulted in expression changes of three nociceptor-enriched voltage-gated

sodium channels, $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$ [19]. Accordingly, miRNA expressions are globally changed in various pain states in the DRG, spinal cord, and brain regions such as the limbic system and prefrontal cortex. However, chronic pain can arise from a variety of pathologies, including damage to the somatosensory system, cancers, musculoskeletal and visceral disorders and genetic diseases, and consequently the miRNA expression profiles are highly distinctive depending on the causes. In this chapter, we discuss the roles of miRNAs in pain based on the underlying pathologies.

miRNAs in Acute Pain

Acute pain is evoked by a wide range of nociceptive stimuli, including incisions and exposure to irritants, and is rarely sustained for a long time. Postoperative incisional pain is a common form of acute pain associated with clinical concern. Skin incisions penetrating the underlying muscle were reported to downregulate miR-203 expression in keratinocytes [20]. Substance P, a well-known neuropeptide involved in pain transmission, mediated the miR-203 downregulation after an incision. Phospholipase A2-activating protein in keratinocytes was identified as a candidate target of miR-203 to cause mechanical allodynia following an incision. Thus, peripherally released substance P may act on keratinocytes to upregulate phospholipase A2-activating protein through miR-203 downregulation, and may be involved in incisional pain.

Noxious stimuli associated with various chemical irritants were shown to acutely affect miRNA expressions and pain behaviors. miR-1 was detected in nearly all DRG neurons [21], and was upregulated in the DRG, but not in the dorsal spinal cord, at 10 min after subcutaneous injection of capsaicin, an agonist for the heat-activated receptor TRPV1 [22]. Axotomy also increased miR-1 expression in the DRG at 1 day postoperatively, while other painful procedures, such as subcutaneous complete Freund's adjuvant (CFA) injection and partial sciatic nerve ligation, decreased its expression within 1 day [22]. Interestingly, bone cancer increased miR-1 expression at 8 days after tumor cell implantation, while inhibition of miR-1 function suppressed the mechanical hypersensitivity associated with bone cancer [23]. Although several pain-related genes are predicted to be targets of miR-1 [22], it remains unclear how these targets contribute to an acute pain state as well as bone cancer pain. Besides miR-1, subcutaneous capsaicin injection increased miR-16 expression in the DRG, but decreased it in the dorsal spinal cord [22]. Although miR-16 is predicted to target several pain-related genes, such as brain derived-neurotrophic factor (BDNF) [24], mitogen-activated protein kinase (MAPK) 3 [25], and TRPC3 [26], the actual contribution of miR-16 to pain remains unknown.

Intraplantar formalin injection, which is commonly used to induce acute spontaneous pain behaviors, was shown to decrease miR-124a expression in putative nociceptive spinal neurons and non-nociceptive DRG neurons [27]. MeCP2, a transcriptional regulator involved in inflammatory pain [28, 29], appears to be an

important miR-124 target gene. The 3′-UTR of MeCP2 mRNA was directly targeted by miR-124, as examined by luciferase assays. MeCP2 expression, which was observed in spinal cells expressing miR-124, was upregulated following formalin or miR-124 inhibitor treatment, but downregulated following miR-124 mimic treatment. Furthermore, BDNF, which promotes nociceptive transmission in the dorsal horn, is a well-known MeCP2 target gene, and was downregulated by intrathecal administration of an miR-124 mimic in intact mice. Accordingly, intrathecal administration of a miR-124 mimic reduced the second phase of formalin-induced pain. On the other hand, intrathecal miR-124 treatment was shown to alleviate persistent or chronic pain induced by carrageenan injection and peripheral nerve injury [30], although it was not clarified whether miR-124 expression was reduced in these pain conditions. Thus, miR-124 downregulation appears to be involved not only in acute pain, but also in chronic pain.

miRNAs also contribute to acute pain through unconventional mechanisms of action. Park et al. [31] showed that let-7b was released from cultured DRG neurons upon acute neuronal excitation with a variety of stimuli including formalin, capsaicin, and ionomycin. Extracellular let-7b excited nociceptor neurons through binding to Toll-like receptor (TLR) 7, which is activated by single-stranded RNAs to initiate innate immune responses [32], with subsequent activation of the cold-activated receptor TRPA1 on nociceptive neurons. Accordingly, intraplantar injection of let-7b elicited rapid spontaneous pain, while injection of its inhibitor reduced formalin-induced spontaneous pain. Thus, besides their well-known roles in translational modulation, miRNAs could be released from DRG neurons and induce rapid excitation of nociceptive neurons as signal transmitters for peripheral and spinal neurons as well as microglia and astrocytes, thereby contributing to sensitization of the nociceptive pathway.

miRNAs in Neuropathic Pain: The Peripheral Nervous System

Lesions or diseases of the somatosensory system such as the primary afferents and spinal cord cause intractable neuropathic pain that can become chronic. In clinical practice, DRG and TG neurons are the principal origins of neuropathic pain observed in traumatic injury, herpes zoster, diabetes, or cancer chemotherapy, and their functional changes are involved in both the initiation and maintenance of chronic neuropathic pain [33].

Microarray and deep-sequencing analyses revealed massive changes in miRNA expressions in the DRG following a variety of peripheral nerve injuries, including nerve transection [34–41], chronic constriction injury (CCI) [42], nerve crush [43], spared nerve injury [44], and spinal nerve ligation [45–47]. Nerve injury also changed the miRNA expressions in the proximal and/or distal sciatic nerves, which possibly reflect the miRNA expressions in the axons of DRG neurons and/or Schwann cells [36, 48, 49]. Several miRNA expression levels in serum were also reported to be changed [50, 51]. Although peripheral nerve injury causes massive

changes in miRNAs, the functional relevance of a large proportion of these miRNAs remains putative. Notably, the dicer-miRNA pathway is critical for peripheral nerve regeneration and functional recovery in vivo and regenerative axonogenesis in vitro [52]. Some miRNAs that have been validated in pain behaviors are reviewed below.

miR-1 expression in the DRG varies depending on the type of peripheral nerve injury. Kusuda et al. [22] first showed that miR-1 was downregulated in the DRG after partial sciatic nerve ligation, but upregulated after axotomy of the sciatic nerve. Norcini et al. [44] showed that sural nerve injury downregulated miR-1 expression in the DRG, while tibial nerve injury upregulated its expression. They suggested that miR-1 downregulation is involved in the development of chronic pain based on their findings that sural nerve injury induced chronic neuropathic pain, while tibial nerve injury induced transient neuropathic pain. As described earlier, miR-1 expressions were also upregulated by capsaicin injection, axotomy, and bone cancer [22, 23]. Furthermore, inhibition of miR-1 suppressed bone cancer pain [23]. Therefore, the roles of miR-1 in pain appear to be complicated and variable depending on the causes of the pain. On the other hand, miR-1 may also be involved in recovery from nerve injury because transfection of an miR-1 mimic into cultured DRG neurons attenuated neurite outgrowth [21].

miR-7a plays a key role in the late phase of neuropathic pain through regulation of neuronal excitability in DRG neurons [45]. miR-7a was mainly expressed in small cell-sized neurons, which are considered to be nociceptive neurons. Following spinal nerve ligation, miR-7a expression was decreased in DRG neurons only in the late phase of neuropathic pain. Adeno-associated virus (AAV) serotype 6 vector-mediated overexpression of miR-7a in DRG neurons suppressed the maintenance, but not the development, of neuropathic pain, while functional blockade of miR-7a in intact rats caused pain-related behaviors. On the other hand, miR-7a overexpression had no effect on acute or CFA-induced inflammatory pain, consistent with the lack of expression changes in miR-7a observed in inflammatory pain. As a responsible target of miR-7a, the β2 subunit of voltage-gated sodium channels, which affects the cell surface expression of these channels and leads to increased neuronal excitability [53, 54], was identified. β2 subunit expression was increased by nerve injury or miR-7a blockade. Accordingly, miR-7a normalized the hyperexcitability of nociceptive DRG neurons in the neuropathic pain state.

miR-182-96-183 is a miRNA cluster that includes three distinct miRNAs (miR-182, miR-96, and miR-183) located close to one another on the genome, and is possibly transcribed as a single common primary transcript. All three cluster members were highly enriched in the DRG and were downregulated in DRG neurons after spinal nerve ligation [55]. Accordingly, miR-96 and miR-183 were shown to suppress neuropathic pain induced by traumatic peripheral nerve injury [56, 57]. miR-96 was decreased in parallel with $Na_V1.3$ upregulation in the DRG after nerve injury. Intrathecal administration of miR-96 suppressed $Na_V1.3$ expression in association with alleviation of neuropathic pain [56]. Similarly, miR-183 was decreased in association with increased $Na_V1.3$ and BDNF mRNA expressions. Intrathecal administration of a lentiviral vector encoding miR-183 suppressed $Na_V1.3$ and BDNF mRNA expressions as well as neuropathic pain [57]. It remains unknown whether

these miRNAs directly target $Na_V1.3$ and BDNF. $Na_V1.3$, BDNF, substance P, and voltage-dependent calcium channel subunit α2δ1 were predicted to be targets for these cluster members in silico [55].

miR-21 is one of the well-characterized oncogenic miRNAs upregulated in almost all kinds of carcinoma cells [58]. miR-21 also has a wide range of physiological and pathological functions, including roles in the immune system and cardiovascular diseases [59]. In the DRG, miR-21 was consistently upregulated in almost all neurons after various nerve injuries, including axotomy [34, 35], CCI [60], nerve crush [48], and spinal nerve injury [60]. Continued miR-21 upregulation was observed, even at 6 months after sciatic nerve denervation [40]. Intrathecal administration of an miR-21 inhibitor suppressed the late phase of neuropathic pain [60]. On the other hand, miR-21 expression in the DRG was unchanged in an inflammatory pain condition induced by CFA injection (unpublished data). Conversely, serum miR-21 expression was decreased in rats after spinal nerve ligation [50]. In DRG neurons, miR-21 was found to be involved in cellular responses to neuronal damage as well as neuropathic pain. miR-21 enhanced axonal growth of DRG neurons in vitro [34] and inhibited apoptosis of cultured DRG neurons [61], suggesting that miR-21 contributes to functional recovery from nerve injury. Furthermore, the molecular mechanisms underlying miR-21 regulation have been investigated. miR-21 expression was upregulated in the DRG following intrathecal injection of interleukin (IL)-1β [60] and in cultured DRG neurons treated with IL-6 [61]. Meanwhile, nerve growth factor, a well-known neurotrophic factor for a subpopulation of DRG neurons, increased miR-21 expression in PC12 cells [62].

miRNAs in Neuropathic Pain: The Spinal Cord

Peripheral nerve injury affects miRNA expressions not only in DRG neurons, but also in neurons and glial cells in the spinal cord and brain. Although these cells were spared direct injury, robust synaptic inputs and cytokine signaling from primary afferents were established to affect these spinal cells to induce synaptic plasticity and glial activation [10]. Accordingly, changes in miRNA expressions have been reported in the dorsal spinal cord in peripheral neuropathy caused by traumatic peripheral nerve injury [42, 63, 64] or streptozotocin-induced diabetes [65].

miR-103 was the first well-characterized miRNA in the neuropathic pain context [66]. Those authors found that all three subunits, Cav1.2-α1, Cav1.2-α2δ1, and Cav1.2-β1, comprising $Ca_V1.2$ L-type calcium channels, which underlie the long-term plastic changes in chronic neuropathic pain [67], were direct targets for miR-103. Indeed, miR-103 functionally modulated calcium transients in cultured spinal neurons. Spinal nerve injury decreased miR-103 expression, which was enriched in neurons in superficial laminae of the dorsal horn, and increased the mRNA expressions of calcium channel subunits. Intrathecal application of miR-103 reduced the increased calcium channel expressions. In line with these results, intrathecal miR-103 application alleviated neuropathic pain.

Neuroinflammation is a consistently observed phenomenon after neuronal damage and its development in the spinal dorsal horn is critically associated with hyperalgesia. Spinal glial cells, microglia, and astrocytes were activated in a variety of chronic pain states and released cytokines to facilitate nociceptive transmission [10]. miR-155, which is intensively involved in the regulation of inflammation-associated diseases [68], was upregulated in the spinal cord following CCI [69] and in osteoarthritis [70], although the spinal cells expressing miR-155 remain unknown. Intrathecal administration of an miR-155 inhibitor attenuated neuropathic pain and suppressed the increases in proinflammatory cytokine expressions, including IL-1β and IL-6. Increases in IκBα protein expression and p38 MAPK phosphorylation following CCI were also suppressed by the miR-155 inhibitor. As a candidate target, suppressor of cytokine signaling 1 (SOCS1) was shown to be directly suppressed by miR-155. Accordingly, SOCS1 was decreased in the spinal cord and this decrease was reversed by intrathecal miR-155 inhibition.

Autophagy, a critical regulator of neuroinflammation [71], was found to be dysregulated in the spinal cord following peripheral nerve injury and intrathecal injection of an autophagy inhibitor, chloroquine, induced mechanical hyperalgesia [72]. Accordingly, miR-195 was reported to mediate neuroinflammation and neuropathic pain through autophagy regulation [73]. miR-195 was upregulated in the dorsal spinal cord and isolated spinal microglia after spinal nerve ligation. Putative autophagic activity assessed by LC3 expression in isolated spinal microglia was decreased by spinal nerve ligation and increased by an miR-195 inhibitor. In primary cultures of spinal microglia, miR-195 enhanced the induction of two proinflammatory cytokines, IL-1β and tumor necrosis factor-α, after treatment with lipopolysaccharide, although miR-195 alone could not induce these cytokine expressions. Intrathecal administration of a miR-195 inhibitor suppressed neuropathic pain and the analgesic effect was impaired by another autophagy inhibitor, 3-methyladenine. ATG14, an important regulator of autophagy [74], was identified as a direct target of miR-195. Thus, miR-195 upregulation appears to contribute to neuropathic pain via enhancement of neuroinflammation by autophagy inhibition.

miR-203 expression was decreased in the dorsal spinal cord, but not in the DRG, hippocampus or anterior cingulate cortex, following bilateral CCI [42, 75]. The Rap1a 3′-UTR was identified as a target of miR-203 in vitro, although the actual target sequence was not determined [75]. In addition, Rap1a was increased in the dorsal spinal cord following bilateral CCI. Because Rap1a is a Ras family member involved in synaptic plasticity [76] and was previously shown to be increased in the spinal cord following noxious stimulation with formalin [77], miR-203 may be involved in neuropathic pain development [75].

Spinal cord injury is another clinical condition associated with neuropathic pain and causes global miRNA expression changes [78–82]. Although some miRNAs were shown to be involved in key processes of spinal cord injury that lead to functional deficits including cell death, inflammation, and astrogliosis [83], the involvement of these miRNA expression changes in neuropathic pain is largely unknown. miR-124 was found to be decreased [79, 80] or unchanged [84, 85] in the spinal cord following spinal cord injury. Intermittent electrical stimulation of the tail

decreased miR-124 expression in the contused dorsal spinal cord of rats [85]. Given that intrathecal miR-124 treatment reduced formalin- and carrageenan-induced pain and neuropathic pain caused by peripheral nerve injury as described above, miR-124 may also alleviate neuropathic pain caused by spinal cord injury.

miRNAs in Inflammation-Induced Superficial Pain

Inflammation that occurs in or just below the skin causes persistent pain in the body surface, which disappears upon dissipation of the inflammation. Peripheral inflammation impacts on the neural and glial functions of the DRG, spinal cord, and brain, similar to the case for neuropathic pain. Microarray and TaqMan array analyses were performed in the TG [86], spinal cord [87], and hippocampus [88] following peripheral tissue inflammation. Notably, DRG neuron-specific deletion of Dicer was shown to attenuate inflammatory pain induced by intraplantar injection of carrageenan and CFA [19], suggesting the importance of miRNAs in inflammatory pain.

miR-124 promotes a quiescent state in microglia [89] and alters the functions of spinal microglia in pain transmission. Willemen et al. [30] showed that hyperalgesia induced by intraplantar injection of the pro-inflammatory cytokine IL-1β caused a shift from a transient pain state to a persistent pain state in microglia/macrophage-specific GRK2 heterozygous (LysM-GRK2$^{+/-}$) mice in association with reduced miR-124 expression in isolated spinal microglia. Intrathecal miR-124 treatment suppressed the increased and decreased marker expressions for pro-inflammatory (M1-type) and anti-inflammatory (M2-type) macrophages, respectively, in the spinal cord. miR-124 prevented the transition to persistent hyperalgesia induced by intraplantar IL-1β in LysM-GRK2$^{+/-}$ mice, although it did not have any effect on the course of hyperalgesia in wild-type mice. On the other hand, miR-124 attenuated inflammatory pain induced by intraplantar carrageenan injection in wild-type mice. Therefore, miR-124 downregulation may be a common causative change in various types of persistent pain conditions.

Epigenetically suppressed expression of miR-219 was reported to regulate inflammatory pain [87]. miR-219 expression was downregulated in spinal neurons after intraplantar CFA injection. Intrathecal administration of an miR-219 mimic or lentiviral vector encoding miR-219 alleviated hyperalgesia in CFA-injected mice, while intrathecal administration of a lentiviral vector encoding an miR-219 sponge (inhibitor) induced hyperalgesia in intact mice. CaMKIIγ was identified as a miR-219 target gene responsible for pain modulation. miR-219 inhibited CaMKIIγ upregulation after CFA injection, while miR-219 inhibition promoted CaMKIIγ expression. In addition, hyperalgesia induced by the miR-219 sponge was suppressed by CaMKIIγ siRNA treatment. Furthermore, NMDAR1 (GluN1), a major CaMKIIγ substrate, was involved in miR-219-CaMKIIγ-dependent hyperalgesia. Interestingly, hypermethylation of the miR-219 promoter played a role in miR-219 downregulation and hyperalgesia, suggesting the importance of epigenetic modulation of miR-219 in the chronicity of inflammatory pain.

Several other miRNAs have been proposed as modulators of inflammatory pain, although their exact contributions to pain remain speculative. miR-134 undergoes biphasic changes in DRG and TG neurons after CFA injection, exhibiting downregulation followed by upregulation [90, 91]. Ni et al. [91] identified μ-opioid receptors, which showed upregulated expression in DRG neurons after CFA injection, as a direct target of miR-134 in vitro. Since many other miRNAs and μ-opioid receptor were reported to regulate each other, miRNAs may be extensively involved in the modulation of opioid analgesia and anti-nociceptive tolerance [92]. On the other hand, miR-125a-3p expression was downregulated in the TG soon after CFA injection into the orofacial skin [86]. Luciferase assays showed that miR-125a-3p targeted the 3′-UTR of the p38 MAPK gene in ND8/34 cells, a hybrid cell line of rat DRG neurons and mouse neuroblastoma. In their study, p38 MAPK protein was upregulated in the TG after CFA injection [86], although the p38 MAPK phosphorylation level, but not the protein level, was shown to increase and cause inflammatory pain [93]. Finally, miR-183, a member of the miR-182-96-183 cluster reportedly involved in neuropathic pain as described above, was also downregulated in the TG after CFA injection [86, 90].

miRNAs in Arthritic Pain

Arthritis is a joint disease that manifests severe pain as a major symptom. Osteoarthritis is the most prevalent degenerative joint disease associated with progressive loss of articular cartilage. Rheumatoid arthritis is a chronic, immune-mediated, inflammatory disease that primarily affects joints. Expression changes in a variety of miRNAs were reported in the joints and validated in the pathogenesis of both forms of arthritis [94–97]. However, the miRNA expressions in the somatosensory system in arthritis are largely unknown.

Using microarray analyses, Li et al. [70] reported the miRNA expression changes in the spinal cord of rats with osteoarthritis induced by medial meniscus transection. Among the miRNAs in the microarray, miR-146a downregulation was validated by quantitative PCR in the spinal cord as well as in the DRG, although the miR-146a-expressing cell types were not determined. Expression changes in miR-146a were also reported in other painful conditions. miR-146a was increased in the spinal cord following spinal cord injury [81, 84, 85], but decreased in DRG neurons in a diabetic peripheral neuropathy model [98]. Microarray analyses showed that miR-146a was upregulated in the DRG after sciatic nerve axotomy [35] and spinal nerve injury [45]. miR-146a is known to play roles in innate immune responses and inflammation [99, 100] and is downregulated in affected cartilage in osteoarthritis patients [101, 102]. Accordingly, a miR-146a mimic suppressed lipopolysaccharide- or IL-1β-induced upregulation of inflammatory mediators including proinflammatory cytokines (IL-1β and IL-6) and TLRs in BV2 cells, a microglial cell line, and primary cultured astrocytes. Interestingly, a single nucleotide polymorphism of miR-146a was suggested to be associated with susceptibility to juvenile idiopathic arthritis-enthesitis-related

arthritis in an Indian population [103]. Therefore, miR-146a may provide an efficient means to suppress glia-mediated neuroinflammation, although the contribution of miR-146a per se to pain caused by arthritis remains unknown.

miRNAs in Visceral Pain

Visceral pain arises from an enormous range of diseases associated with inflammation, mechanical stresses, cancers, or unknown causes. Microarray or TaqMan array analyses revealed miRNA dysregulation in colonic biopsies [104] and whole-blood samples [105] from patients with irritable bowel syndrome (IBS) and bladder biopsies from patients with bladder pain syndrome (BPS) [106]. However, reports on the involvement of miRNAs in visceral pain have been limited thus far to a few chronic pain syndromes [107].

miR-199a and miR-199b were downregulated in colonic biopsies from patients with diarrhea-predominant IBS and the miR-199a expression level was inversely correlated with the visceral pain score [104]. TRPV1 was upregulated in the colon of patients with IBS and was identified as a target of miR-199a in vitro. miR-199a expression was also decreased in the colon and DRG of rats with intracolonic infusion of 2,4,6-trinitrobenzene sulfonic acid, a well-established colitis model with visceral hypersensitivity. Intraperitoneal injection of a lentiviral vector encoding miR-199a attenuated nociceptive responses to colonic distension in association with TRPV1 reduction in the DRG and colon. In contrast to the downregulation in IBS, miR-199a was upregulated in BPS [106]. miR-199a was expressed in the bladder smooth muscles as well as in urothelial cells [108]. miR-199a directly targeted mRNAs encoding LIN7C, ARHGAP12, PALS1, RND1, and PVRL1 and regulated intercellular junctions of urothelial cells.

miR-29a was increased in the blood microvesicles, duodenum, and colon of IBS patients with increased intestinal membrane permeability [109]. miR-29a enhanced epithelial permeability in human epithelial cell lines from the colon and small intestine. Glutamine synthase, which was downregulated in IBS patients, was directly targeted by miR-29a. Glutamine synthase catalyzes the conversion of ammonia and glutamate to glutamine and decreased levels of intestinal glutamine synthetase are thought to lead to low levels of available glutamine and increased intestinal membrane permeability. Since siRNA-mediated glutamine synthase inhibition also increased the epithelial permeability in vitro and a glutamine supplement reversed the increased intestinal permeability in IBS, miR-29a plausibly modulates increased intestinal permeability in IBS.

In chronic visceral pain caused by zymosan-induced neonatal cystitis, a dozen miRNAs were shown to be modulated in the dorsal spinal cord of adult rats [110]. Among them, miR-181 was upregulated in the spinal cells of adult rats with neonatal cystitis, while the $GABA_A$ receptor α1 subunit, a target of miR-181, was downregulated. Accordingly, oblique muscle responses to colorectal distension in adult rats with neonatal saline treatment and heightened muscle responses in adult rats with adult zymosan treatment were attenuated by intrathecal injection of a $GABA_A$

receptor agonist, muscimol, while heightened muscle responses in adult rats with neonatal zymosan treatment were unaffected. The authors suggested that miRNA-mediated downregulation of $GABA_A$ receptors following neonatal cystitis resulted in loss of inhibitory tone in the adult spinal cord, leading to long-lasting visceral hypersensitivity.

TaqMan array analyses for miRNA expression profiles showed that 28 miRNAs were upregulated and three miRNAs were downregulated in bladder dome biopsies from patients with BPS [106]. Four upregulated miRNAs (miR-320, miR-328, miR-449b, and miR-550) were shown to decrease NK_1 receptor expression levels and/or SP-induced intracellular calcium increases. Accordingly, NK_1 receptor expression was decreased in the bladder dome of patients with BPS, although the physiological significance of NK_1 receptor downregulation in BPS remains unclear.

miRNAs in Cancer Pain

Although miRNA functions have been intensively investigated in cancers, the involvement of miRNAs in cancer pain is poorly understood. In bone cancer pain caused by injection of tumor cells into the calcaneus bone, 57 miRNAs were shown to be changed by more than 2.5-fold in the DRG using microarray analyses [23]. Three upregulated miRNAs were examined to elucidate their functional significance. Intrathecal injection of inhibitors for miR-1a-3p or miR-34c-5p attenuated the mechanical hypersensitivity, while inhibition of miR544-3p was ineffective. On the other hand, three downregulated miRNAs showed distinct effects on bone cancer pain.

miR-370-3p and miR-483-3p mimics enhanced and inhibited, respectively, the mechanical allodynia accompanying bone cancer, while miR-291b-5p had no effects. Chloride channel, voltage-sensitive 3 (CLCN3) was expressed in DRG neurons including peptidergic and non-peptidergic putative nociceptive neurons, and played roles in regulating the amount of neurotransmitter and the release probability of synaptic vesicles in hippocampal glutamatergic neurons [111]. CLCN3, a direct target of miR-1a-3p, was downregulated after tumor cell implantation and upregulated by an miR-1a-3p inhibitor. Although the detailed relationship between CLCN3 and cancer pain is unknown, knockdown of CLCN3 by siRNA injection enhanced cancer pain. Given that miR-1a-3p was also shown to be upregulated in the DRG after axotomy and capsaicin injection [22], miR-1a-3p may be additionally involved in pain conditions other than cancer.

miRNAs in Other Pain Conditions

miRNA expression changes have been reported in body fluid samples from patients with other intractable pain conditions, although their pathological significance is uncertain. In patients with complex regional pain syndrome, the miRNA expression

profiles in blood samples were examined for their correlations with pain levels, comorbidities, and medications [112]. Four miRNAs (miR-296-5p, miR-361-3p, miR-532-3p, and miR-30d) were found to be correlated with pain levels, while many other miRNAs were correlated with comorbidities such as high blood pressure, high cholesterolemia, headache, thyroid disease, and hypoesthesia. Other miRNAs were correlated with use of narcotics and antiepileptic medications, while miR-192 was correlated with both thyroid disease and use of epileptic drugs.

Regarding patients with fibromyalgia, the miRNA expression profiles in their cerebrospinal fluid and serum have been reported [113, 114]. In cerebrospinal fluid collected from the lumbar region of female patients, nine miRNAs including miR-21 and miR-195 were significantly decreased compared with healthy controls. Among them, miR-145-5p was positively correlated with the levels of pain and fatigue [114]. In serum samples collected from female patients, seven miRNAs were significantly lower and one miRNA was higher compared with those from healthy women. Several miRNAs were correlated with pain levels or sleep quality, and notably miR-103a-3p was correlated with both pain and sleep quantity [113].

Finally, the miRNA expression profiles in serum samples from patients with migraine were compared between attacks and pain-free periods, and the comparisons revealed that 32 miRNAs were aberrantly expressed [115]. Among the top four miRNAs with the highest fold changes, miR-382-5p and possibly miR-29c-5p were also increased during pain-free periods compared with matched healthy controls.

Brain miRNAs in Pain Conditions

Pain significantly impacts on higher brain functions, including cognitive, mood and anxiety disorders [116]. In fact, functional MRI studies in humans indicated altered activities in many brain regions in individuals with chronic pain [4]. Pain-related changes were even documented at the single-cell level in the brain [117]. Therefore, through modulation of cellular functions, miRNAs potentially underlie the altered brain functions in pain conditions. Indeed, miRNA expressions were reportedly changed in multiple brain regions in chronic pain conditions, although the functional significance of miRNAs remains largely unknown.

The nucleus accumbens is thought to be involved in the rewarding of pain relief and motivation [118, 119]. In the nucleus accumbens, miR-200b and miR-429 expressions were decreased in the neuropathic pain state with a concomitant increase in the protein expression of DNA methyltransferase 3a (DNMT3a), a putative target for these miRNAs [120]. The authors suggested that increased DNMT3a associated with decreased miRNA-200b/429 clusters in the nucleus accumbens may produce negative emotions along with dysfunction of the "mesolimbic motivation/valuation circuitry." In the hippocampus, the expressions of many miRNAs, such as miR-132 and miR-125b, were changed in neuropathic and inflammatory pain conditions [88, 121]. Interestingly, a wide range of chronic pain conditions with different pathogeneses may share similar miRNA dysregulation at the higher central nervous system level because the modulated miRNAs markedly overlapped one another in neuropathic and

inflammatory pain conditions. In the prefrontal cortex, miR-155 and miR-223 expressions were reported to increase after facial carrageenan injection [122]. Meanwhile, let-7 family members (let-7a, let-7c, and let-7g) were shown to increase in the mouse brain after chronic morphine treatment [123], although the distribution of the miRNA expressions in the brain was undetermined. On the other hand, the let-7 family members directly targeted μ-opioid receptors. Intracerebroventricular treatment with a let-7 inhibitor attenuated the antinociceptive tolerance that developed with chronic use of morphine. Therefore, let-7 upregulation in the brain may mediate opioid tolerance by targeting μ-opioid receptors.

Table 3.1 summarizes miRNAs involved in several pain-related conditions.

Other Noncoding RNAs in Pain

Long noncoding RNAs that exceed 200 nucleotides have also been implicated in neuronal development and diseases including neuropathic pain through transcriptional regulation [124]. Microarray analyses of long noncoding RNAs were performed in the DRG of rats with sciatic nerve resection and revealed that 105 long noncoding RNAs were differentially expressed [125]. A long noncoding RNA, BC089918, was downregulated in DRG neurons after axotomy. BC089918 knockdown with siRNAs promoted neurite outgrowth of cultured DRG neurons. The antisense RNA of potassium channel, voltage-gated shaker-related subfamily A, member 2 (*Kcna2*) mRNA is a well-characterized long noncoding RNA that contributes to neuropathic pain by silencing Kcna2 in primary afferent neurons [126]. A Kcna2 antisense RNA was expressed in DRG neurons containing low amounts of Kcna2 protein. Kcna2 mRNA and protein were downregulated following nerve injury, while Kcna2 antisense RNA was upregulated, and these changes predominantly occurred in large DRG neurons. Accordingly, Kcna2 antisense RNA overexpression in the intact DRG decreased Kcna2 mRNA and protein expressions. Kcna2-related currents recorded from freshly dissociated DRG neurons were found to be reduced by Kcna2 antisense RNA overexpression. In addition, Kcna2 antisense RNA overexpression increased neuronal excitability, with increased resting membrane potential, reduced current threshold for spikes, and increased number of action potentials evoked by electrical stimulation. Finally, Kcna2 antisense RNA overexpression produced mechanical and cold hypersensitivities, while a Kcna2 sense fragment attenuated neuropathic pain by blocking the antisense RNA upregulation.

miRNA Potential for Clinical Application in Pain

In animal experiments, functional modulation of miRNAs has been revealed to suppress pain in various diseases. Given the conservation of miRNAs and their target sequences among species and several results from human samples, miRNAs can be

Table 3.1 Characterized miRNAs in various painful conditions

miRNA	Model	Region	Cell type	Expression change	Contribution to pain	Relevant function	Validated functional target	References
Acute pain								
let-7b	Formalin	Skin	Neuron	–	Yes	Neuronal activation	TLR7 (unconventional)	[31]
miR-124a	Formalin	Spinal cord	Neuron	Down	Unclear	Inflammation	MeCP2	[27]
		DRG	Myelinated neuron	Down	Unclear	–	Unknown	
	Intraplantar IL-1β in LysM-GRK2$^{+/-}$ mice	Spinal cord	Microglia	Down	Yes	Inflammation	Unknown	[30]
miR-203	Incision	Skin	Keratinocyte	Down	N/D	–	Phospholipase A2 activating protein	[20]
Neuropathic pain								
miR-7a	SNL	DRG	Neuron	Down	Yes	Neuronal excitability	Na_V β2	[45]
	CCI				N/D			
miR-21	SCI	Spinal cord	Astrocyte	Up	N/D	Astrocytic hypertrophy	Unknown	[16]
	Fibromyalgia (human)	CSF	–	Down	Not correlated	–	–	[114]
	Axotomy	DRG	Neuron	Up	N/D	–	–	[40]
	SNL	DRG	Neuron	Up	Yes	–	Unknown	[60]
	CCI	DRG		Up	N/D		–	
	SCI	Spinal cord	Neuron	Up (early)	N/D	–	–	[80]
				Down (late)				
	Axotomy	DRG	Neuron	Up	N/D	Axonal growth	Sprouty2	[34]
	SCI	Spinal cord	N/D	Up	N/D	–	–	[84]
	SCI	Spinal cord	N/D	Up	N/D	–	–	[85]
	Nerve crush	Sciatic nerve	Axon	Up	N/D	–	–	[48]
	Axotomy	DRG	N/D	Up	N/D	–	–	[35]
	SCI	Spinal cord	N/D	Up	N/D	–	–	[81]
	SNL	Serum	–	Down	N/D	–	–	[50]
	Axotomy	DRG	N/D	Up	N/D	Apoptosis	–	[61]

miR-96	SNL	DRG	Neuron	Down	N/D	–	–	[55]
	CCI	DRG	N/D	Down	Yes	–	$Na_V1.3$	[57]
miR-103	Fibromyalgia (human)	Serum	–	Down	Correlated	–	–	[113]
	SNL	Spinal cord	Neuron	Down	Yes	Neuronal calcium transient	$Ca_V1.2$-α1, -α2δ1, β1 subunits	[66]
miR-124a	SCI	Spinal cord	N/D	Down	N/D	–	–	[79]
	SCI	Spinal cord	Neuron	Down	N/D	–	–	[80]
	SCI	Spinal cord	N/D	Unchanged	Correlated	–	–	[84]
	SCI	Spinal cord	N/D	Unchanged	N/D	–	–	[85]
	SNI	–	–	N/D	Yes	Inflammation	Unknown	[30]
	Nerve crush	Sciatic nerve	N/D	Down	N/D	–	–	[48]
miR-155	CCI	Spinal cord	N/D	Up	Yes	Inflammation	SOCS1	[69]
miR-183	SNL	DRG	Neuron	Down	N/D	–	–	[55]
	SNL	DRG	N/D	Down	Yes	–	$Na_V1.3$, BDNF	[57]
miR-195	SNL	Spinal cord	Microglia	Up	Yes	Autophagy, inflammation	ATG14	[73]
miR-203	Bilateral CCI	Spinal cord	N/D	Down	N/D	–	Rap1a	[75]
Inflammatory pain								
miR-124a	CFA	TG	N/D	Down (early)	N/D	–	–	[90]
				Up (late)				
	Carrageenan	–	–	N/D	Yes	Inflammation	Unknown	[30]
miR-219	CFA	Spinal cord	Neuron	Down	Yes	–	CaMKIIγ	[87]

(continued)

Table 3.1 (continued)

miRNA	Model	Region	Cell type	Expression change	Contribution to pain	Relevant function	Validated functional target	References
Arthritic pain								
miR-146a	Medial meniscus transection	DRG	N/D	Down	N/D	Inflammation	Unknown	[70]
		Spinal cord	N/D	Down	N/D		–	
	Osteoarthritis (human)	Cartilage	N/D	Down	N/D	Inflammation	Unknown	[101]
	Osteoarthritis (human)	Cartilage	Chondrocyte	Down	N/D	–	Unknown	[102]
Visceral pain								
miR-181	Zymosan-induced neonatal cystitis	Spinal cord	N/D	Up	N/D	–	$GABA_A$ receptor α1 subunit	[110]
miR-199a	Colitis	Colon, DRG	N/D	Down	Yes	–	TRPV1	[104]
	IBS (human)	Colon	N/D	Down	Correlated	–		
	BPS (human)	Bladder dome	N/D	Up	N/D	–	–	[106]
Cancer pain								
miR-1a-3p	Bone cancer	DRG	N/D	Up	Yes	–	CLCN3	[23]
Other noncoding RNA								
Kcna2 antisense RNA	SNL	DRG	Neuron	Up	Yes	Neuronal excitability	Kcna2	[126]

N/D not determined, *CCI* chronic constriction injury, *SCI* spinal cord injury, *SNI* spared nerve injury, *SNL* spinal nerve injury

attractive molecular targets for pain medication. However, major obstacles, such as drug delivery, must be overcome for the clinical development of miRNA-related analgesics.

Oral or transdermal administration is well tolerated and preferable in most patients on cancer pain management, as recommended by the WHO. However, the current miRNA-related drugs are not available as oral or transdermal formulations and require fundamental innovations, although chemical modifications have improved the stability of nucleic acid medicines. Furthermore, even if miRNA-related drugs can enter the systemic circulation, it is not expected that the miRNAs will be delivered in sufficient amounts to sensory neurons, especially central nervous system neurons. In this point, gene transfer may be a preferable means for long-term treatment of chronic pain, although safety remains a critical concern [127]. Modified lentivirus [128], AAV [45, 129], and herpes simplex virus [130] vectors were shown to be efficiently transduced to sensory neurons and to persistently express their transgenes. In addition, gene therapy can be applied in a site-specific manner to avoid systemic adverse effects. On the other hand, exosomes, as naturally occurring membranous microvesicles carrying RNAs, represent another vehicle for RNA medicines. Exosomes are formed and secreted by numerous types of cells and can be transported to other cells. In fact, systemic injection of modified exosomes was reported to deliver the contained molecules, including siRNAs, to neurons, microglia, and oligodendrocytes in the mouse brain [131]. In addition, injection of exosomes derived from macrophages attenuated thermal hyperalgesia and swelling induced by intraplantar CFA injection [132], suggesting the therapeutic potential of exosomes. Overall, the development of an appropriate drug delivery system is a critical issue for clinical application of miRNA-related analgesics.

References

1. Prescott SA, Ma Q, De Koninck Y. Normal and abnormal coding of somatosensory stimuli causing pain. Nat Neurosci. 2014;17:183–91.
2. Kuner R. Central mechanisms of pathological pain. Nat Med. 2010;16:1258–66.
3. Todd AJ. Neuronal circuitry for pain processing in the dorsal horn. Nat Rev Neurosci. 2010; 11:823–36.
4. Apkarian AV, Hashmi JA, Baliki MN. Pain and the brain: specificity and plasticity of the brain in clinical chronic pain. Pain. 2011;152:S49–64.
5. Dworkin RH, O'Connor AB, Kent J, Mackey SC, Raja SN, Stacey BR, Levy RM, Backonja M, Baron R, Harke H, Loeser JD, Treede RD, Turk DC, Wells CD. Interventional management of neuropathic pain: NeuPSIG recommendations. Pain. 2013;154:2249–61.
6. Luo C, Kuner T, Kuner R. Synaptic plasticity in pathological pain. Trends Neurosci. 2014;37: 343–55.
7. Waxman SG, Zamponi GW. Regulating excitability of peripheral afferents: emerging ion channel targets. Nat Neurosci. 2014;17:153–63.
8. Nickel FT, Seifert F, Lanz S, Maihöfner C. Mechanisms of neuropathic pain. Eur Neuropsychopharmacol. 2012;22:81–91.
9. Descalzi G, Ikegami D, Ushijima T, Nestler EJ, Zachariou V, Narita M. Epigenetic mechanisms of chronic pain. Trends Neurosci. 2015;38(4):237–46.

10. Ji RR, Berta T, Nedergaard M. Glia and pain: is chronic pain a gliopathy? Pain. 2013;154: S10–28.
11. McWilliams LA, Cox BJ, Enns MW. Mood and anxiety disorders associated with chronic pain: an examination in a nationally representative sample. Pain. 2003;106:127–33.
12. Quintero GC. Advances in cortical modulation of pain. J Pain Res. 2013;6:713–25.
13. Bushnell MC, Čeko M, Low LA. Cognitive and emotional control of pain and its disruption in chronic pain. Nat Rev Neurosci. 2013;14:502–11.
14. Follert P, Cremer H, Béclin C. MicroRNAs in brain development and function: a matter of flexibility and stability. Front Mol Neurosci. 2014;7:5.
15. Sun E, Shi Y. MicroRNAs: small molecules with big roles in neurodevelopment and diseases. Exp Neurol. 2015;268:46–53.
16. Bhalala OG, Srikanth M, Kessler JA. The emerging roles of microRNAs in CNS injuries. Nat Rev Neurol. 2013;9:328–39.
17. Sakai A, Suzuki H. Emerging roles of microRNAs in chronic pain. Neurochem Int. 2014;77: 58–67.
18. Wang Z. miRNA in the regulation of ion channel/transporter expression. Compr Physiol. 2013;3:599–653.
19. Zhao J, Lee MC, Momin A, Cendan CM, Shepherd ST, Baker MD, Asante C, Bee L, Bethry A, Perkins JR, Nassar MA, Abrahamsen B, Dickenson A, Cobb BS, Merkenschlager M, Wood JN. Small RNAs control sodium channel expression, nociceptor excitability, and pain thresholds. J Neurosci. 2010;30:10860–71.
20. Sun Y, Li XQ, Sahbaie P, Shi XY, Li WW, Liang DY, Clark JD. miR-203 regulates nociceptive sensitization after incision by controlling phospholipase A2 activating protein expression. Anesthesiology. 2012;117:626–38.
21. Bastian I, Tam TS, Zhou XF, Kazenwadel J, Van der Hoek M, Michael MZ, Gibbins I, Haberberger RV. Differential expression of microRNA-1 in dorsal root ganglion neurons. Histochem Cell Biol. 2011;135:37–45.
22. Kusuda R, Cadetti F, Ravanelli MI, Sousa TA, Zanon S, De Lucca FL, Lucas G. Differential expression of microRNAs in mouse pain models. Mol Pain. 2011;7:17.
23. Bali KK, Selvaraj D, Satagopam VP, Lu J, Schneider R, Kuner R. Genome-wide identification and functional analyses of microRNA signatures associated with cancer pain. EMBO Mol Med. 2013;5:1740–58.
24. Merighi A, Salio C, Ghirri A, Lossi L, Ferrini F, Betelli C, Bardoni R. BDNF as a pain modulator. Prog Neurobiol. 2008;85:297–317.
25. Galan A, Lopez-Garcia JA, Cervero F, Laird JMA. Activation of spinal extracellular signaling-regulated kinase-1 and -2 by intraplantar carrageenan in rodents. Neurosci Lett. 2002; 322:37–40.
26. Staaf S, Oerther S, Lucas G, Mattsson JP, Ernfors P. Differential regulation of TRP channels in a rat model of neuropathic pain. Pain. 2009;144:187–99.
27. Kynast KL, Russe OQ, Möser CV, Geisslinger G, Niederberger E. Modulation of central nervous system-specific microRNA-124a alters the inflammatory response in the formalin test in mice. Pain. 2013;154:368–76.
28. Géranton SM, Fratto V, Tochiki KK, Hunt SP. Descending serotonergic controls regulate inflammation-induced mechanical sensitivity and methyl-CpG-binding protein 2 phosphorylation in the rat superficial dorsal horn. Mol Pain. 2008;4:35.
29. Géranton SM, Morenilla-Palao C, Hunt SP. A role for transcriptional repressor methyl-CpG-binding protein 2 and plasticity-related gene serum- and glucocorticoid-inducible kinase 1 in the induction of inflammatory pain states. J Neurosci. 2007;27:6163–73.
30. Willemen HL, Huo XJ, Mao-Ying QL, Zijlstra J, Heijnen CJ, Kavelaars A. MicroRNA-124 as a novel treatment for persistent hyperalgesia. J Neuroinflammation. 2012;9:143.
31. Park CK, Xu ZZ, Berta T, Han Q, Chen G, Liu XJ, Ji RR. Extracellular microRNAs activate nociceptor neurons to elicit pain via TLR7 and TRPA1. Neuron. 2014;82:47–54.
32. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140:805–20.
33. Devor M. Sodium channels and mechanisms of neuropathic pain. J Pain. 2006;7:S3–12.

34. Strickland IT, Richards L, Holmes FE, Wynick D, Uney JB, Wong LF. Axotomy-induced miR-21 promotes axon growth in adult dorsal root ganglion neurons. PLoS One. 2011;6, e23423.
35. Yu B, Zhou S, Qian T, Wang Y, Ding F, Gu X. Altered microRNA expression following sciatic nerve resection in dorsal root ganglia of rats. Acta Biochim Biophys Sin (Shanghai). 2011;43: 909–15.
36. Yu B, Zhou S, Wang Y, Ding G, Ding F, Gu X. Profile of microRNAs following rat sciatic nerve injury by deep sequencing: implication for mechanisms of nerve regeneration. PLoS One. 2011;6, e24612.
37. Zhang HY, Zheng SJ, Zhao JH, Zhao W, Zheng LF, Zhao D, Li JM, Zhang XF, Chen ZB, Yi XN. MicroRNAs 144, 145, and 214 are down-regulated in primary neurons responding to sciatic nerve transection. Brain Res. 2011;1383:62–70.
38. Zhou S, Yu B, Qian T, Yao D, Wang Y, Ding F, Gu X. Early changes of microRNAs expression in the dorsal root ganglia following rat sciatic nerve transection. Neurosci Lett. 2011;494:89–93.
39. Zhou S, Shen D, Wang Y, Gong L, Tang X, Yu B, Gu X, Ding F. microRNA-222 targeting PTEN promotes neurite outgrowth from adult dorsal root ganglion neurons following sciatic nerve transection. PLoS One. 2012;7:e44768.
40. Rau CS, Jeng JC, Jeng SF, Lu TH, Chen YC, Liliang PC, Wu CJ, Lin CJ, Hsieh CH. Entrapment neuropathy results in different microRNA expression patterns from denervation injury in rats. BMC Musculoskelet Disord. 2010;11:181.
41. Lu A, Huang Z, Zhang C, Zhang X, Zhao J, Zhang H, Zhang Q, Wu S, Yi X. Differential expression of microRNAs in dorsal root ganglia after sciatic nerve injury. Neural Regen Res. 2014;9:1031–40.
42. Li H, Shen L, Ma C, Huang Y. Differential expression of miRNAs in the nervous system of a rat model of bilateral sciatic nerve chronic constriction injury. Int J Mol Med. 2013;32: 219–26.
43. Wu D, Murashov AK. MicroRNA-431 regulates axon regeneration in mature sensory neurons by targeting the Wnt antagonist *Kremen1*. Front Mol Neurosci. 2013;6:35.
44. Norcini M, Sideris A, Martin Hernandez LA, Zhang J, Blanck TJJ, Recio-Pinto E. An approach to identify microRNAs involved in neuropathic pain following a peripheral nerve injury. Front Neurosci. 2014;8:266.
45. Sakai A, Saitow F, Miyake N, Miyake K, Shimada T, Suzuki H. miR-7a alleviates the maintenance of neuropathic pain through regulation of neuronal excitability. Brain. 2013;136: 2738–50.
46. von Schack D, Agostino MJ, Murray BS, Li Y, Reddy PS, Chen J, Choe SE, Strassle BW, Li C, Bates B, Zhang L, Hu H, Kotnis S, Bingham B, Liu W, Whiteside GT, Samad TA, Kennedy JD, Ajit SK. Dynamic changes in the microRNA expression profile reveal multiple regulatory mechanisms in the spinal nerve ligation model of neuropathic pain. PLoS One. 2011;6, e17670.
47. Bali KK, Hackenberg M, Lubin A, Kuner R, Devor M. Sources of individual variability: miRNAs that predispose to neuropathic pain identified using genome-wide sequencing. Mol Pain. 2014;10:22.
48. Wu D, Raafat M, Pak E, Hammond S, Murashov AK. MicroRNA machinery responds to peripheral nerve lesion in an injury-regulated pattern. Neuroscience. 2011;190:386–97.
49. Viader A, Chang LW, Fahrner T, Nagarajan R, Milbrandt J. MicroRNAs modulate Schwann cell response to nerve injury by reinforcing transcriptional silencing of dedifferentiation-related genes. J Neurosci. 2011;31:17358–69.
50. Xu Y, Zhang X, Pu S, Wu J, Lv Y, Du D. Circulating microRNA expression profile: a novel potential predictor for chronic nervous lesions. Acta Biochim Biophys Sin (Shanghai). 2014;46:942–9.
51. Metpally RPR, Nasser S, Malenica I, Courtright A, Carlson E, Ghaffari L, Villa S, Tembe W, Van Keuren-Jensen K. Comparison of analysis tools for miRNA high throughput sequencing using nerve crush as a model. Front Genet. 2013;4:20.
52. Wu D, Raafat A, Pak E, Clemens S, Murashov AK. *Dicer*-microRNA pathway is critical for peripheral nerve regeneration and functional recovery in vivo and regenerative axonogenesis in vitro. Exp Neurol. 2012;233:555–65.

53. Isom LL, Ragsdale DS, De Jongh KS, Westenbroek RE, Reber BFX, Scheuer T, Catterall WA. Structure and function of the β2 subunit of brain sodium channels, a transmembrane glycoprotein with a CAM motif. Cell. 1995;83:433–42.
54. Lopez-Santiago LF, Pertin M, Morisod X, Chen C, Hong S, Wiley J, Decosterd I, Isom LL. Sodium channel β2 subunits regulate tetrodotoxin-sensitive sodium channels in small dorsal root ganglion neurons and modulate the response to pain. J Neurosci. 2006;26:7984–94.
55. Aldrich BT, Frakes EP, Kasuya J, Hammond DL, Kitamoto T. Changes in expression of sensory organ-specific microRNAs in rat dorsal root ganglia in association with mechanical hypersensitivity induced by spinal nerve ligation. Neuroscience. 2009;164:711–23.
56. Chen HP, Zhou W, Kang LM, Yan H, Zhang L, Xu BH, Cai WH. Intrathecal miR-96 inhibits Nav1.3 expression and alleviates neuropathic pain in rat following chronic construction injury. Neurochem Res. 2014;39:76–83.
57. Lin CR, Chen KH, Yang CH, Huang HW, Sheen-Chen SM. Intrathecal miR-183 delivery suppresses mechanical allodynia in mononeuropathic rats. Eur J Neurosci. 2014;39:1682–9.
58. Selcuklu SD, Donoghue MTA, Spillane C. *miR-21* as a key regulator of oncogenic processes. Biochem Soc Trans. 2009;37:918–25.
59. Kumarswamy R, Volkmann I, Thum T. Regulation and function of miRNA-21 in health and disease. RNA Biol. 2011;8:706–13.
60. Sakai A, Suzuki H. Nerve injury-induced upregulation of miR-21 in the primary sensory neurons contributes to neuropathic pain in rats. Biochem Biophys Res Commun. 2013;435:176–81.
61. Zhou S, Zhang S, Wang Y, Yi S, Zhao L, Tang X, Yu B, Gu X, Ding F. miR-21 and miR-222 inhibit apoptosis of adult dorsal root ganglion neurons by repressing TIMP3 following sciatic nerve injury. Neurosci Lett. 2015;586:43–9.
62. Mullenbrock S, Shah J, Cooper GM. Global expression analysis identified a preferentially nerve growth factor-induced transcriptional program regulated by sustained mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) and AP-1 protein activation during PC12 cell differentiation. J Biol Chem. 2011;286:45131–45.
63. Genda Y, Arai M, Ishikawa M, Tanaka S, Okabe T, Sakamoto A. microRNA changes in the dorsal horn of the spinal cord of rats with chronic constriction injury: A TaqMan® Low Density Array study. Int J Mol Med. 2013;31:129–37.
64. Brandenburger T, Castoldi M, Brendel M, Grievink H, Schlösser L, Werdehausen R, Bauer I, Hermanns H. Expression of spinal cord microRNAs in a rat model of chronic neuropathic pain. Neurosci Lett. 2012;506:281–6.
65. Gong Q, Lu Z, Huang Q, Ruan L, Chen J, Liang Y, Wang H, Yue Y, Feng S. Altered microRNAs expression profiling in mice with diabetic neuropathic pain. Biochem Biophys Res Commun. 2015;456:615–20.
66. Favereaux A, Thoumine O, Bouali-Benazzouz R, Roques V, Papon MA, Salam SA, Drutel G, Leger C, Calas A, Nagy F, Landry M. Bidirectional integrative regulation of Cav1.2 calcium channel by microRNA miR-103: role in pain. EMBO J. 2011;30:3830–41.
67. Fossat P, Dobremez E, Bouali-Benazzouz R, Favereaux A, Bertrand SS, Kilk K, Léger C, Cazalets JR, Langel Ü, Landry M, Nagy F. Knockdown of L calcium channel subtypes: differential effects in neuropathic pain. J Neurosci. 2010;30:1073–85.
68. Vigorito E, Kohlhaas S, Lu D, Leyland R. miR-155: an ancient regulator of the immune system. Immunol Rev. 2013;253:146–57.
69. Tan Y, Yang J, Xiang K, Tan Q, Guo Q. Suppression of MicroRNA-155 attenuates neuropathic pain by regulating SOCS1 signalling pathway. Neurochem Res. 2014;40:550–60.
70. Li X, Kroin JS, Kc R, Gibson G, Chen D, Corbett GT, Pahan K, Fayyaz S, Kim JS, van Wijnen AJ, Suh J, Kim SG, Im HJ. Altered spinal microRNA-146a and the microRNA-183 cluster contribute to osteoarthritic pain in knee joints. J Bone Miner Res. 2013;28:2512–22.
71. Arroyo DS, Gaviglio EA, Peralta Ramos JM, Bussi C, Rodriguez-Galan MC, Iribarren P. Autophagy in inflammation, infection, neurodegeneration and cancer. Int Immunopharmacol. 2014;18:55–65.
72. Berliocchi L, Maiarù M, Varano GP, Russo R, Corasaniti MT, Bagetta G, Tassorelli C. Spinal autophagy is differently modulated in distinct mouse models of neuropathic pain. Mol Pain. 2015;11:3.

73. Shi G, Shi J, Liu K, Liu N, Wang Y, Fu Z, Ding J, Jia L, Yuan W. Increased miR-195 aggravates neuropathic pain by inhibiting autophagy following peripheral nerve injury. Glia. 2013;61:504–12.
74. Obara K, Ohsumi Y. Atg14: a key player in orchestrating autophagy. Int J Cell Biol. 2011;2011:713435.
75. Li H, Huang Y, Ma C, Yu X, Zhang Z, Shen L. MiR-203 involves in neuropathic pain development and represses Rap1a expression in nerve growth factor differentiated neuronal PC12 cells. Clin J Pain. 2015;31:36–43.
76. Stornetta RL, Zhu JJ. Ras and Rap signaling in synaptic plasticity and mental disorders. Neuroscientist. 2011;17:54–78.
77. Urayama O, Murakoshi T, Kubota M, Hara Y, Chihara J, Ikawa Y. Coincident induction of K *rev*-1/*rap* 1A, *rap* 1B and H-*ras* mRNAs in the rat spinal cord by noxious stimulation. Brain Res Mol Brain Res. 1997;45:331–4.
78. Liu NK, Wang XF, Lu QB, Xu XM. Altered microRNA expression following traumatic spinal cord injury. Exp Neurol. 2009;219:424–9.
79. Nakanishi K, Nakasa T, Tanaka N, Ishikawa M, Yamada K, Yamasaki K, Kamei N, Izumi B, Adachi N, Miyaki S, Asahara H, Ochi M. Responses of microRNAs 124a and 223 following spinal cord injury in mice. Spinal Cord. 2010;48:192–6.
80. Strickland ER, Hook MA, Balaraman S, Huie JR, Grau JW, Miranda RC. MicroRNA dysregulation following spinal cord contusion: implications for neural plasticity and repair. Neuroscience. 2011;186:146–60.
81. Yunta M, Nieto-Díaz M, Esteban FJ, Caballero-Lopez M, Navarro-Ruíz R, Reigada D, Pita-Thomas DW, del Águila Á, Muñoz-Galdeano T, Maza RM. MicroRNA dysregulation in the spinal cord following traumatic injury. PLoS One. 2012;7, e34534.
82. Hu JZ, Huang JH, Zeng L, Wang G, Cao M, Lu HB. Anti-apoptotic effect of microRNA-21 after contusion spinal cord injury in rats. J Neurotrauma. 2013;30:1349–60.
83. Nieto-Diaz M, Esteban FJ, Reigada D, Muñoz-Galdeano T, Yunta M, Caballero-López M, Navarro-Ruiz R, del Águila Á, Maza RM. MicroRNA dysregulation in spinal cord injury: causes, consequences, and therapeutics. Front Cell Neurosci. 2014;8:53.
84. Strickland ER, Woller SA, Hook MA, Grau JW, Miranda RC. The association between spinal cord trauma-sensitive miRNAs and pain sensitivity, and their regulation by morphine. Neurochem Int. 2014;77:40–9.
85. Strickland ER, Woller SA, Garraway SM, Hook MA, Grau JW, Miranda RC. Regulatory effects of intermittent noxious stimulation on spinal cord injury-sensitive microRNAs and their presumptive targets following spinal cord contusion. Front Neural Circuits. 2014;8:117.
86. Dong Y, Li P, Ni Y, Zhao J, Liu Z. Decreased microRNA-125a-3p contributes to upregulation of p38 MAPK in rat trigeminal ganglions with orofacial inflammatory pain. PLoS One. 2014;9, e111594.
87. Pan Z, Zhu LJ, Li YQ, Hao LY, Yin C, Yang JX, Guo Y, Zhang S, Hua L, Xue ZY, Zhang H, Cao JL. Epigenetic modification of spinal miR-219 expression regulates chronic inflammation pain by targeting CaMKIIγ. J Neurosci. 2014;34:9476–83.
88. Hori Y, Goto G, Arai-Iwasaki M, Ishikawa M, Sakamoto A. Differential expression of rat hippocampal microRNAs in two rat models of chronic pain. Int J Mol Med. 2013;32:1287–92.
89. Ponomarev ED, Veremeyko T, Barteneva N, Krichevsky AM, Weiner HL. MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-α-PU.1 pathway. Nat Med. 2011;17:64–70.
90. Bai G, Ambalavanar R, Wei D, Dessem D. Downregulation of selective microRNAs in trigeminal ganglion neurons following inflammatory muscle pain. Mol Pain. 2007;3:15.
91. Ni J, Gao Y, Gong S, Guo S, Hisamitsu T, Jiang X. Regulation of μ-opioid type 1 receptors by microRNA134 in dorsal root ganglion neurons following peripheral inflammation. Eur J Pain. 2013;17:313–23.
92. Barbierato M, Zusso M, Skaper SD, Giusti P. MicroRNAs: emerging role in the endogenous μ opioid system. CNS Neurol Disord Drug Targets. 2015;14:239–50.
93. Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. Neuron. 2002;36:57–68.

94. Ammari M, Jorgensen C, Apparailly F. Impact of microRNAs on the understanding and treatment of rheumatoid arthritis. Curr Opin Rheumatol. 2013;25:225–33.
95. Barter MJ, Young DA. Epigenetic mechanisms and non-coding RNAs in osteoarthritis. Curr Rheumatol Rep. 2013;15:353.
96. Picascia A, Grimaldi V, Pignalosa O, De Pascale MR, Schiano C, Napoli C. Epigenetic control of autoimmune diseases: from bench to bedside. Clin Immunol. 2015;157:1–15.
97. Yu C, Chen WP, Wang XH. MicroRNA in osteoarthritis. J Int Med Res. 2011;39:1–9.
98. Wang L, Chopp M, Szalad A, Zhang Y, Wang X, Zhang RL, Liu XS, Jia L, Zhang ZG. The role of miR-146a in dorsal root ganglia neurons of experimental diabetic peripheral neuropathy. Neuroscience. 2014;259:155–63.
99. Chan EKL, Ceribelli A, Satoh M. MicroRNA-146a in autoimmunity and innate immune responses. Ann Rheum Dis. 2013;72:ii90–5.
100. Montagner S, Orlandi EM, Merante S, Monticelli S. The role of miRNAs in mast cells and other innate immune cells. Immunol Rev. 2013;253:12–24.
101. Jones SW, Watkins G, Le Good N, Roberts S, Murphy CL, Brockbank SMV, Needham MRC, Read SJ, Newham P. The identification of differentially expressed microRNA in osteoarthritic tissue that modulate the production of TNF-α and MMP13. Osteoarthritis Cartilage. 2009;17:464–72.
102. Yamasaki K, Nakasa T, Miyaki S, Ishikawa M, Deie M, Adachi N, Yasunaga Y, Asahara H, Ochi M. Expression of microRNA-146a in osteoarthritis cartilage. Arthritis Rheum. 2009;60:1035–41.
103. Singh S, Rai G, Aggarwal A. Association of microRNA-146a and its target gene IRAK1 polymorphism with enthesitis related arthritis category of juvenile idiopathic arthritis. Rheumatol Int. 2014;34:1395–400.
104. Zhou Q, Yang L, Larson S, Basra S, Merwat S, Tan A, Croce C, Verne GN. Decreased miR-199 augments visceral pain in patients with IBS through translational upregulation of TRPV1. Gut. 2015, in press.
105. Fourie NH, Peace RM, Abey SK, Sherwin LB, Rahim-Williams B, Smyser PA, Wiley JW, Henderson WA. Elevated circulating miR-150 and miR-342-3p in patients with irritable bowel syndrome. Exp Mol Pathol. 2014;96:422–5.
106. Sanchez Freire V, Burkhard FC, Kessler TM, Kuhn A, Draeger A, Monastyrskaya K. MicroRNAs may mediate the down-regulation of neurokinin-1 receptor in chronic bladder pain syndrome. Am J Pathol. 2010;176:288–303.
107. Gheinani AH, Burkhard FC, Monastyrskaya K. Deciphering microRNA code in pain and inflammation: lessons from bladder pain syndrome. Cell Mol Life Sci. 2013;70:3773–89.
108. Monastyrskaya K, Sánchez-Freire V, Hashemi GA, Klumpp DJ, Babiychuk EB, Draeger A, Burkhard FC. miR-199a-5p regulates urothelial permeability and may play a role in bladder pain syndrome. Am J Pathol. 2013;182:431–48.
109. Zhou Q, Souba WW, Croce CM, Verne GN. MicroRNA-29a regulates intestinal membrane permeability in patients with irritable bowel syndrome. Gut. 2010;59:775–84.
110. Sengupta JN, Pochiraju S, Kannampalli P, Bruckert M, Addya S, Yadav P, Miranda A, Shaker R, Banerjee B. MicroRNA-mediated $GABA_{A\alpha-1}$ receptor subunit down-regulation in adult spinal cord following neonatal cystitis-induced chronic visceral pain in rats. Pain. 2013;154:59–70.
111. Guzman RE, Alekov AK, Filippov M, Hegermann J, Fahlke C. Involvement of ClC-3 chloride/proton exchangers in controlling glutamatergic synaptic strength in cultured hippocampal neurons. Front Cell Neurosci. 2014;8:143.
112. Orlova IA, Alexander GM, Qureshi RA, Sacan A, Graziano A, Barrett JE, Schwartzman RJ, Ajit SK. MicroRNA modulation in complex regional pain syndrome. J Transl Med. 2011;9:195.
113. Bjersing JL, Bokarewa MI, Mannerkorpi K. Profile of circulating microRNAs in fibromyalgia and their relation to symptom severity: an exploratory study. Rheumatol Int. 2014;35(4):635–42.
114. Bjersing JL, Lundborg C, Bokarewa MI, Mannerkorpi K. Profile of cerebrospinal microRNAs in fibromyalgia. PLoS One. 2013;8, e78762.

115. Andersen HH, Duroux M, Gazerani P. Serum microRNA signatures in migraineurs during attacks and in pain-free periods. Mol Neurobiol. 2015, in press.
116. Tracey I, Mantyh PW. The cerebral signature for pain perception and its modulation. Neuron. 2007;55:377–91.
117. Saab CY. Pain-related changes in the brain: diagnostic and therapeutic potentials. Trends Neurosci. 2012;35:629–37.
118. Baliki MN, Geha PY, Fields HL, Apkarian AV. Predicting value of pain and analgesia: nucleus accumbens response to noxious stimuli changes in the presence of chronic pain. Neuron. 2010;66:149–60.
119. Navratilova E, Porreca F. Reward and motivation in pain and pain relief. Nat Neurosci. 2014;17: 1304–12.
120. Imai S, Saeki M, Yanase M, Horiuchi H, Abe M, Narita M, Kuzumaki N, Suzuki T, Narita M. Change in microRNAs associated with neuronal adaptive responses in the nucleus accumbens under neuropathic pain. J Neurosci. 2011;31:15294–9.
121. Arai M, Genda Y, Ishikawa M, Shunsuke T, Okabe T, Sakamoto A. The miRNA and mRNA changes in rat hippocampi after chronic constriction injury. Pain Med. 2013;14:720–9.
122. Poh KW, Yeo JF, Ong WY. MicroRNA changes in the mouse prefrontal cortex after inflammatory pain. Eur J Pain. 2011;15:801.e1–12.
123. He Y, Yang C, Kirkmire CM, Wang ZJ. Regulation of opioid tolerance by let-7 family microRNA targeting the μ opioid receptor. J Neurosci. 2010;30:10251–8.
124. Clark BS, Blackshaw S. Long non-coding RNA-dependent transcriptional regulation in neuronal development and disease. Front Genet. 2014;5:164.
125. Yu B, Zhou S, Hu W, Qian T, Gao R, Ding G, Ding F, Gu X. Altered long noncoding RNA expressions in dorsal root ganglion after rat sciatic nerve injury. Neurosci Lett. 2013;534:117–22.
126. Zhao X, Tang Z, Zhang H, Atianjoh FE, Zhao JY, Liang L, Wang W, Guan X, Kao SC, Tiwari V, Gao YJ, Hoffman PN, Cui H, Li M, Dong X, Tao YX. A long noncoding RNA contributes to neuropathic pain by silencing Kcna2 in primary afferent neurons. Nat Neurosci. 2013;16: 1024–31.
127. Vannucci L, Lai M, Chiuppesi F, Ceccherini-Nelli L, Pistello M. Viral vectors: a look back and ahead on gene transfer technology. New Microbiol. 2013;36:1–22.
128. Takasu K, Sakai A, Hanawa H, Shimada T, Suzuki H. Overexpression of GDNF in the uninjured DRG exerts analgesic effects on neuropathic pain following segmental spinal nerve ligation in mice. J Pain. 2011;12:1130–9.
129. Pleticha J, Heilmann LF, Evans CH, Asokan A, Samulski RJ, Beutler AS. Preclinical toxicity evaluation of AAV for pain: evidence from human AAV studies and from the pharmacology of analgesic drugs. Mol Pain. 2014;10:54.
130. Goss JR, Krisky D, Wechuck J, Wolfe D. Herpes simplex virus-based nerve targeting gene therapy in pain management. J Pain Res. 2014;7:71–9.
131. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJA. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol. 2011;29:341–5.
132. McDonald MK, Tian Y, Qureshi RA, Gormley M, Ertel A, Gao R, Lopez EA, Alexander GM, Sacan A, Fortina P, Ajit SK. Functional significance of macrophage-derived exosomes in inflammation and pain. Pain. 2014;155:1527–39.

Chapter 4
microRNA and Epilepsy

Cristina R. Reschke and David C. Henshall

Abstract Epilepsy is a common, serious neurological disease characterized by recurring seizures. Such abnormal, excessive synchronous firing of neurons arises in part because of imbalances in excitation and inhibition in the brain. The process of epileptogenesis, during which the normal brain is transformed after injury to one capable of generating spontaneous seizures, is associated with large-scale changes in gene expression. These contribute to the remodelling of brain networks that permanently alters excitability. Components of the microRNA (miRNA) biogenesis pathway have been found to be altered in brain tissue from epilepsy patients and experimental epileptogenic insults result in select changes to miRNAs regulating neuronal microstructure, cell death, inflammation, and ion channels. Targeting key miRNAs has been shown to alter brain excitability and suppress or exacerbate seizures, indicating potential for miRNA-based therapeutics in epilepsy. Altered miRNA profiles in biofluids may be potentially useful biomarkers of epileptogenesis. In summary, miRNAs represent an important layer of gene expression control in epilepsy with therapeutic and biomarker potential.

Keywords Biomarker • Epileptogenesis • Hippocampus • Neurodegeneration • Status epilepticus

Introduction

Epilepsy is the most common neurological disease of childhood and adolescence and the second most common in adulthood after stroke. It is a chronic disease characterized by recurrent seizures, which are caused by sudden, usually brief, excessive electrical discharges in a group of neurons in the brain. The clinical manifestation of a seizure can vary from a brief lapse of attention or muscle jerks, to severe and

C.R. Reschke • D.C. Henshall, Ph.D. (✉)
Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin, Ireland
e-mail: dhenshall@rcsi.ie

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_4

prolonged convulsions. Several mechanisms underlying the process that transforms the normal brain into one that generates spontaneous recurrent seizures are known [1, 2]. However, much remains unknown about the observed changes as cause or consequence of epilepsy, and how they mutually interact. Identifying the involved pathways and the molecular mechanisms coordinating gene expression is crucial to a better understanding of the pathogenic process and to develop new treatment approaches.

Seizures in a majority of patients can be successfully prevented with antiepileptic drugs (AED) but nearly one-third of patients are resistant to currently available therapies. Novel treatments are urgently needed for this patient group. In addition, we need anti-epileptogenic treatments that can prevent epilepsy developing in at-risk patients, for example, following insults to the brain that lead to epilepsy (e.g., traumatic brain injury). Or a treatment which modifies the disease's natural course, slowing progression and alleviating symptoms rather than acting simply to suppress seizures as do conventional AEDs.

microRNAs (miRNA) are an endogenous class of small noncoding RNAs that play an important role in multiple biological processes via posttranscriptional control of gene expression. They mainly function to reduce protein levels in cells through sequence-specific binding to target mRNAs, leading to transcript degradation or translational repression. The brain has an abundant and diverse contingent of miRNAs, which are crucial for normal development, and establishment and maintenance of cell phenotype. Acute and chronic nervous system diseases, including epilepsy, are associated with mis-regulation of key components of the miRNA biogenesis pathway and altered expression of miRNA. The first reports on epilepsy and miRNA appeared in 2010, followed by large-scale miRNA profiling studies characterizing the alterations in miRNA expression in experimental and human epilepsy. More recently, functional studies in rodents have shown single miRNAs can exert powerful effects on brain excitability, seizures, and epilepsy.

The present chapter provides an overview on epilepsy followed by a focus on the main discoveries on miRNA biogenesis and expression in experimental and human epilepsy. Thereafter, functional studies of epilepsy-related miRNAs will be described focusing on those with proven effects in promoting epilepsy and candidate drug and/or targets for prevention or modification of the disease.

Epilepsy

Epilepsy is a brain disease characterized by an enduring predisposition to generate seizures, and by neurological, cognitive, and psychological consequences inherent of this condition [3, 4]. It is estimated that around 65 million people worldwide have epilepsy [5]. Seizures are the hallmarks of epilepsy, and have been defined by the International League Against Epilepsy (ILAE) as "*a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the*

brain" [6]. Seizures are believed to result from an imbalance between excitatory and inhibitory neurotransmission. It may be focally limited to an area or cerebral hemisphere (focal epileptic seizures) or appear bilaterally (generalized epileptic seizures) [4]. Clinically, focal seizures present features such as discrete motor movements, automatisms, altered awareness, and responsiveness. Generalized seizures are related with tonic-clonic, atonic, myoclonic and absence seizures, as well as loss of consciousness [3].

Most seizures last only a few minutes or less, terminating due to endogenous anticonvulsant mechanisms in the brain (which are themselves poorly understood). *Status epilepticus* (SE) is a life-threatening, neurologic emergency previously defined as "*seizure that persists for a sufficient length of time or is repeated frequently enough that recovery between attacks does not occur*" [7]. It has been shown that seizures that do not spontaneously terminate within 5–10 min are unlikely to end without pharmacological intervention [8, 9]. Therefore, SE has been clinically defined as a seizure longer than 5 min or more than one seizure within a 5 min period without recovery of consciousness [10]. If seizures persist for 30 min or more this can produce irreversible brain injury [11].

Epilepsies are often classified according to their causes as: (1) genetic, where genetic factors have a major causal role in the disease (with Mendelian or complex patterns of inheritance or the result of de novo mutations); (2) structural or metabolic, where acquired epilepsy is the result of changes in neuronal network excitability that follow an initial precipitating insult to the brain (i.e., traumatic brain injury (TBI), stroke, tumor, developmental malformations, febrile seizures); and (3) unknown or idiopathic, where no cause has been identified [12, 13].

Epidemiology

The incidence of epilepsy shows significant differences between developing compared with developed countries. This is due to the high levels of parasitic infections, untreated childhood epilepsy, and problems during childbirth [14]. In Africa and South America the highest incidence of epilepsy is in young adults [4]. In developed countries, epilepsy is more common in children (congenital) and the elderly (associated with chronic conditions, such as tumors and Alzheimer's disease) [15].

There does not appear to be a strong sex difference in the risk of epilepsy, although there is a lack of consistency among the majority of incidence studies. Generally, incidence of epilepsy or of unprovoked seizures is more prevalent in males than in females (50.7 vs. 46.2 per 100,000, respectively) [16, 17]. It has been reported that males are more predisposed to develop partial epilepsies [18, 19], and it is associated with the higher prevalence of lesional epilepsy in men than women [18]. In contrast, cryptogenic temporal lobe epilepsy (TLE) seems to be more prevalent in females [18, 19]. Recent reviews suggest these differences only exist in some forms of epilepsy [20], and are not prominent.

Diagnosis

Epilepsy is not always easily diagnosed. An accurate diagnosis requires a clear differentiation between seizures and other factors that may alter consciousness, and discrimination between spontaneous unprovoked seizures from provoked seizures. It is necessary to classify seizure type and epilepsy syndrome, determining etiology where possible, age of onset of seizure, EEG patterns and brain imaging profiles, and associated behavior. Prognosis can vary widely from benign syndromes which resolve in later childhood to life-threatening epileptic encephalopathies. While a delay in the diagnosis of authentic epilepsy seldom causes problems, a false-positive diagnosis may have serious implications for the patient [21]. Unfortunately, cases of misdiagnosis are common worldwide—around 20–30 % of patients. Aiming to reduce these rates, an ILAE Task Force formulated conceptual definitions of "seizure" and "epilepsy" [6], which have been recently adapted into a global concept and translated into practical definitions [3]. It has been proposed that epilepsy is a brain disease defined by any of the following conditions: "(1) *At least two unprovoked seizures occurring more than 24 h apart;* (2) *one unprovoked seizure and a probability of further seizures similar to the general recurrence risk (at least 60 %) after two unprovoked seizures, occurring over the next 10 years;* (3) *diagnosis of an epilepsy syndrome*." Epilepsy might be considered resolved (but not necessarily cured) for patients who either had an age-dependent epilepsy syndrome but are now past the applicable age, or who have remained seizure- and medication-free for the last 10 and 5 years, respectively [3]. An epilepsy diagnosis does not obligatorily require treatment. Conversely, the decision for treatment may be prophylactic considering predisposition for more seizures.

Treatment

Phenobarbital was introduced in 1912 as the first modern antiseizure medication followed by phenytoin. Second generation treatments included carbamazepine, acetazolamide, and sodium valproate and were introduced between 1950s and 1960s. A further surge in new AEDs, the so-called third generation, appeared in the 1990s. These included vigabatrin, lamotrigine, topiramate, levetiracetam, oxcarbazepine, zonisamide, gabapentin, pregabalin, and lacosamide.

Generally, around 70 % of patients with epilepsy achieve seizure freedom with the first prescribed antiseizure medication. Choice of first-line drug takes into account factors such as seizure types and syndrome (focal vs. generalized), age and sex (childbearing age, pregnancy, breast-feeding), and drug interactions (i.e., patients with learning disability or under other medications). The majority of AEDs share one or more mechanism of action in common. These are enhancing inhibitory (γ-amino butyric acid; GABA) transmission, suppressing excitatory signaling, or blocking voltage-dependent ion channels. However, AEDs with other mechanisms

of action are known, including levetiracetam which targets part of the synaptic vesicle release machinery [22]. Perampanel, which acts as a selective noncompetitive antagonist of AMPA receptors, is the most recently licensed adjunctive drug [23].

For the remaining ~30 % of patients who are refractory to the currently available drug treatments (one or more drugs) [24] some may be suitable for non-pharmacological treatments, such as surgical resection and vagus nerve stimulation (VNS). Unfortunately, surgical removal of the epileptic focus is only suitable for a minority of the patients. The efficacy of brain stimulation is also suboptimal. VNS was mainly developed as a palliative treatment for those patients, and only 5 % of the patients achieve seizure freedom [25].

Experimental Models of Epilepsy

For complete reviews on experimental models of epilepsy the reader is referred elsewhere [26–29]. Animal models of epilepsy and status epilepticus have been enormously helpful to understand neuronal mechanisms of normal and abnormal brain function [30], explore new diagnostic approaches, or to test new therapeutic targets. There is a wide range of different experimental models available (in vitro and in vivo) with selection based on the particular questions being asked. Summarily, animal models can be divided in: (1) acute seizure models—in which an acute ictal activity is provoked by an insult (i.e., chemical or electrical), allowing researchers to investigate the fundamental basis of ictal discharges, effects of seizures on the brain, and seizure termination; (2) chronic epilepsy models—which mimic epilepsy (mainly TLE), and are relevant to study interictal activity and potential mechanisms (i.e., excitotoxicity and synaptic reorganization, etc.). In this section, we will briefly present some of the most widely used models.

Kainic Acid

Kainic acid (KA) is a neurotoxin [31] and structural analogue of glutamate, which preferentially binds and activates the KA subtype of ionotropic glutamate receptor [32]. These receptors are abundantly present in the hippocampus (mainly in the CA3 region), amygdala, perirhinal, and entorhinal cortex [33]. Systemic administration of KA leads to synchronous firing of neurons within the limbic system that typically develops into status epilepticus. Intrahippocampal [34] and intra-amygdala [35] delivery are also common approaches to trigger seizures which avoids exposure of the whole brain to the toxin and produces more restricted lesions within the hippocampus. KA models are considered an important tool to study complex partial seizures with secondary generalization [4, 36] as well as model epileptogenesis [37, 38]. The prolonged seizures typically produce hippocampal pathology with similarities to that seen in patients with mesial temporal sclerosis, including loss of

excitatory and inhibitory neurons and gliosis. Among limitations of this model is the sometimes poor correlation between motor and electroencephalographic (EEG) activity, and the inter-strain variability in seizure and histopathological responses [39, 40].

Pilocarpine

Pilocarpine is a cholinergic muscarinic agonist that is usually delivered by systemic injection into rodents. Pilocarpine elicits seizures through metabotropic acetylcholine receptors, which are expressed in the hippocampus, striatum, and cortex [41]. However, research suggests that pilocarpine first causes a local inflammation process and consequent blood–brain barrier (BBB) disruption, which contributes to the CNS hyperexcitability [42–44]. Seizures induced by pilocarpine have limbic features including facial automatisms and tonic-clonic components which become long-lasting and develop into SE. Pilocarpine-induced SE typically produces damage to the CA1 and hilar region of the hippocampus but also extensive extra-hippocampal pathology and often high mortality [40].

Pentylenetetrazole

Pentylenetetrazole (PTZ) is a GABAergic antagonist which induces short-lasting seizures by binding to the benzodiazepine and picrotoxin sites of the $GABA_A$ receptor and blocking endogenous inhibitory control in the brain. The drug is given in one or more systemic injections, and elicits stereotypical behaviors (freezing, myoclonic twitches, clonic seizures, and tonic-clonic generalized seizures) and corresponding EEG patterns (i.e., epileptiform spiking). The PTZ model has been popular for screening putative AEDs but is also suitable for studies of the effects of single seizures on the brain. Other $GABA_A$ antagonists (e.g., bicuculline and picrotoxin) are also used occasionally to produce seizures, working via the same mechanism [40].

Kindling

Kindlingrefers to models in which a progressive and permanent reduction of seizure threshold is generated in response to intermittent and repeated subconvulsant stimuli (electrical or chemical) [27, 45, 46]. Kindling initially models focal partial seizures; however, with the reduction of seizure threshold precipitates the spread of seizures to produce a model of complex partial seizures with secondary generalization [27, 47]. Kindling has been successful at identifying mechanisms of epileptogenesis and also for screening AEDs. It has been reported that spontaneous seizures

can result from the repeated stimulation, but studies have shown conflicting findings and its applicability to human epilepsy is controversial [48].

Mechanisms Underlying Epilepsy

Genetic Mutations

Genetic factors have a major causal role in epilepsy with a number identified which have Mendelian (familial) inheritance patterns. Albeit pure epilepsy syndromes are rare, the study of these mutations has provided great insights into molecular mechanisms of epilepsy [49, 50]. Most familial cases involve mutations in genes encoding ion channels and neurotransmitter receptors. This fits with our current thinking that seizures arise due to an imbalance between excitatory and inhibitory pathways. This does not explain, of course, why seizures are sporadic despite a permanent genetic mutation. An autosomal dominant mutation in the KCNQ2/3 genes is an example of benign familial neonatal epilepsy. Hyperexcitability is increased because of reduced potassium efflux during plasma membrane repolarization [49, 51]. Epileptic encephalopathies are a devastating group of severe childhood epilepsy disorders for which the cause is often unknown. De novo mutations are those present in an affected child only and include epileptic encephalopathies such as Lennox-Gastaut and Dravet syndromes. The majority of Dravet syndrome cases (which are considered an intractable and uncontrollable epilepsy) result from mutations of the *SCN1A* gene which encodes a voltage-gated sodium channel [52]. Nevertheless, mutations have been discovered in genes without obvious links to excitability including topoisomerase II (*TOP2*), which is involved in DNA stress and repair [53], and *ALG13* which encodes an enzyme involved in *N*-linked glycosylation [52]. Interestingly, a number of diseases in which epilepsy is a common comorbidity result from mutations in genes closely related with miRNA function. For example, Fragile-X syndrome, a disease which results in epilepsy in about 15 % of the cases, is a result of mutations in the *FMR1* gene, the product of which interacts with proteins involved in miRNA biogenesis and function [54]. Likewise, deletions of 22q11.2 which includes *DCGR8* (DiGeorge syndrome critical region 8; a microprocessor complex subunit required for miRNA processing) is directly associated with seizures and childhood epilepsy [55, 56].

Acquired Epilepsy

Changes in neuronal network excitability may be a consequence of an initial precipitating insult to the brain. Traumatic brain injury, infection, stroke, tumors, and prolonged febrile seizures are leading causes of acquired epilepsy. TLE is often attributed to an earlier brain insult and is one of the most common forms of epilepsy

in humans. This type of epilepsy is generally accompanied by hippocampal sclerosis, which is histopathologically marked by neuronal cell loss, gliosis, and synaptic reorganization [57, 58]. These changes are thought to be important in the pathogenesis and maintenance of the epileptic state. Here we summarize current concepts on how cell and molecular changes may promote epileptogenesis.

Neuronal Cell Loss

Neuronal cell death is a common feature in mesial TLE patients' hippocampi [59]. It is commonly associated with each stage of the epileptogenesis process [60], particularly when the trigger is a traumatic [61] or other brain insult (e.g., SE). Although select cell death is thought to promote epilepsy, some studies have failed to observe an antiepileptogenic effect of neuroprotection [62, 63]. Loss of cells may promote epilepsy by creating an imbalance between excitatory and inhibitory inhibition [64] or by provoking reactive gliosis and inflammation [2]. In line with this view, seizure-mediated neuronal loss is not essential to generate inflammation, but proinflammatory cytokine release can contribute to cell loss; thus, dying cells may maintain inflammation [2, 65]. Additionally, apoptosis-related signaling pathways are prominently activated in experimental and human TLE supporting a role of cell death in epileptogenesis [66, 67].

Neuroinflammation: Microglia and Astrocyte Activation

There is strong evidence to support the involvement of inflammatory processes in the pathogenesis of seizures and epileptogenesis [2, 68, 69], regardless of an immune-mediated or infectious cause [65]. Expression of genes associated with inflammatory and immune pathways are markedly upregulated in human and experimental TLE [70]. This includes cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6, which are rapidly induced in locally activated astrocytes and microglia [2]. Microglia are the resident immune cells in the CNS and these cells become activated after brain injury or immunological stimuli [71]. Microglia activation has been frequently found in resected brain tissue from patients with epilepsy [68, 72] and after experimental SE in rodents [73, 74]. Microglial activation and the consequent neuroinflammation are also linked to the progression of neurodegeneration [75]. The microglial inflammatory response has also been suggested to promote BBB damage, which further promotes seizures by disrupting ionic balances, neurotransmitters, and cell metabolism [76].

Astrocytes are highly abundant cells in the brain and are co-responsible for CNS homeostasis, exerting functions such as structural support, ionic balance and BBB maintenance, and clearance of neurotransmitters. Human and experimental TLE is invariably associated with astrocytosis within the seizure focus, which is thought to

promote ionic and neurotransmitter imbalances and consequent enhancement of seizure susceptibility [77]. Other pro-inflammatory molecules released by glia include complement factors [78], products of cyclooxygenase (COX)-2 pathway [68, 79, 80], and damage-activated molecular patterns (DAMPS/danger signals) such as high mobility group box 1 (HMGB1) [81]. Seizure-induced brain inflammation can persist for days after seizure termination [2, 65] leading to transcriptional events which may contribute to long-lasting decreases in seizure threshold and further promoting neuroinflammation [2, 65].

Neuroinflammation is not invariably bad for the brain. The brain uses the inflammation process to protect cells by ensuring rapid resolution of tissue damage after injury. For instance, a neuroprotective inflammatory response is generated by purinergic receptor signaling after TBI [82]. Also, stress-hormone-dependent mechanisms trigger an amplification of Th2 and a suppression of Th1 immune responses in the brain, which increases the production of anti-inflammatory cytokines such as IL-10 [68].

BBB Disruption

Experimental models and human studies have linked damage and leakage of the BBB to seizure development [44, 83–85]. Also, it has been shown that the occurrence of seizures and neuroinflammation directly enhance BBB permeability. Summarily, some mechanisms have been proposed to directly link hyperexcitability and epileptic focus to BBB disruption: (1) Astrocyte activation due to blood-derived protein extravasation to the CNS [86, 87]; (2) Inflammation and enhancement of leukocyte adhesion proteins [2, 42–44]; (3) Epileptic activity itself that upregulates the endothelial efflux transporter P-glycoprotein, which is an essential BBB gatekeeper [78, 88].

Neuronal Plasticity

The brain is capable of profound structural changes in response to insults. Structural modifications involving dendritic and axonal reorganization have been reported in hippocampi of experimental and human epileptic tissue (reviewed by [77]). Mossy fiber sprouting (MFS)—the growth of new axon collaterals from granule cells of the dentate gyrus—is a feature observed in tissue from epilepsy patients and experimental models [89–91]. There is also significant evidence of dendritic structural changes in human and rodents hippocampus. In vivo imaging shows both short- and long-term changes to dendritic spines following seizures and this may modulate excitability by adding, adjusting the strength, or removing synaptic contacts [96, 97]. However, although this structural reorganization seems to be common, it is not agreed how important it is for aberrant neuronal networks [92, 93], and remains a

point of some controversy [94, 95]. Recently, experiments using rapamycin showed this blocked MFS but did not stop epileptogenesis in TLE [95]. It remains undecided whether MFS promotes or inhibits the occurrence of spontaneous seizures.

microRNAs and CNS

microRNA Biogenesis and Mechanisms of Action

microRNA (miRNA) is a class of small (~22 nucleotides) non-protein-coding RNA molecules that regulate gene expression either by promoting mRNA degradation or by attenuating protein translation at the posttranscriptional level [96]. Briefly, miRNAs are initially transcribed as primary transcripts (pri-miRNA) by polymerase II (Pol II), processed to pre-miRNA and finally to mature miRNA by the actions of Drosha and Dicer, respectively. The functional strand of the mature miRNA is uploaded into the RNA-induced silencing complex (RISC) which contains the protein Argonaute 2 (Ago-2). The RISC-loaded miRNA is then guided to silence a target mRNA through mRNA degradation or translational inhibition. It is estimated that a third or more mRNAs are regulated by miRNA, particular those with long 3′UTRs (see Chap. 2 of the volume "microRNA: Basic Science" for a detailed discussion of miRNA machinery). A given miRNA can have several binding sites to the same mRNA and a single mRNA may be targeted by multiple miRNAs, thereby producing stronger effects [97]. In line with this view, a single miRNA is able to regulate the expression of possibly hundreds of genes, thereby exerting important effects on cellular functions [98].

Around 50 % of all identified miRNAs are expressed in the mammalian brain [99, 100]. Individual miRNAs display specific cell type and brain region distribution which contributes to their important roles in brain development and function [101–103]. In the brain, miRNAs and their biogenesis components are selectively localized within dendrites in addition to the cell soma in neurons. This allows local and activity-dependent miRNA production and regulation of protein levels at individual synapses [102, 104]. The importance of miRNA in brain development and function is exemplified by studies of mice with constitutive or conditional deletion of Drosha or Dicer [105–109]. Deletion of DGCR8 produces a reduction in brain size and loss of inhibitory synaptic neurotransmission [107, 110]. Ago-2 deficiency in mice results in death during early embryogenesis or mid-gestation [111]. Selective Dicer ablation in the CNS at later embryonic stages strongly impairs migration of late-born neurons in the cortex and oligodendrocyte precursor expansion and differentiation in the spinal cord [112].

miRNA biogenesis components have been found to be altered in experimental and human epilepsy. Whereas Drosha levels were normal in resected hippocampus from TLE patients [113] and mouse hippocampus soon after SE [113, 114], Dicer levels were decreased in some sclerotic hippocampi from experimental and human TLE [113]. Supporting the loss of Dicer as pathogenic, mice lacking Dicer have been found to develop seizures and neurodegeneration [108]. The mechanism which

explains the lower Dicer levels in certain TLE hippocampi is unknown but Dicer is a caspase-3 substrate [115], and caspase-3 is activated in sclerotic hippocampi from TLE patients [116]. Ago-2 protein levels were found to be higher in hippocampus and neocortex of TLE patients [113], although were not altered after SE or TLE in mice [113, 114, 117]. Therefore, alterations in one or more components of the miRNA biogenesis pathway may directly contribute to epileptogenesis or established epilepsy.

miRNAs Regulate Key Processes in Epilepsy

The ability of individual miRNAs to have several targets within the same cell, and thus potentially exert effects on multiple pathways, make them attractive to interrupt or modify epilepsy [118]. Indeed, preclinical tests of compounds targeting single protein-coding genes have largely failed to disrupt the epileptogenesis process [119]. It has been shown that certain miRNAs (e.g., miR-132) control several epileptogenesis-related processes, such as cell death and neuroinflammation [120, 121]. Analysis of gene expression in mice lacking miR-128 revealed alterations to over 100 protein-coding genes including multiple channels and signaling components within the ERK2 signaling network [122]. Recent work has shown that miR-92a regulates AMPA receptor expression [123], and miR-128, miR-132, and miR-134 are implicated in the regulation of dendritic spines, the major contact points for excitatory communication in the CNS [124]. miR-21 and miR-146a target inflammatory components within innate and adaptive immune responses [125, 126]. miR-21 upregulation is induced by inflammatory insults, and several inflammatory targets (i.e., interleukins) were identified [129]. miR-146a was the first inflammation-associated miRNA linked to epilepsy [126]. It is the most consistently upregulated miRNA in epilepsy studies, with enhanced hippocampal levels after SE (experimentally) and in resected hippocampus from intractable TLE patients [126–129]. The mechanism that induces miR-146a level increases is possibly driven by IL-1β and inflammation-associated transcription factors including nuclear factor kappa B [129, 130] (see dedicated Chap. 9 in the volume “microRNA: Basic Science”).

miRNA Profiling in Status Epilepticus and Epilepsy

Gene expression profiling has been a powerful tool to capture the altered molecular environment of the epileptic brain [119]. Profiling studies have investigated relevant pathways linked to the process of epileptogenesis and epilepsy and many are predicted to be under miRNA control [2, 119, 131]. The majority of experimental data come from studies which profiled miRNA responses in rodent hippocampus shortly after SE [114, 132–135]. Combined, studies to date have found altered expression levels of over 100 miRNAs after SE. This number is likely to increase as

genome-wide and RNAseq studies allow more complete coverage of the miRNAome. The large numbers indicate that perhaps 30–50 % of all miRNAs are altered in the epileptic brain. These studies also reveal brain-region specific temporal changes to miRNA expression in the wake of SE (Table 4.1). That is, unique as well as overlapping miRNA expression responses are found within subfields of the hippocampus after SE and in chronic epilepsy [133]. It must be remembered, however, that all miRNA profiling studies to date have used a tissue "pool" of cells so it is difficult to know whether the miRNAs are really functional (uploaded to the RISC) or active in specific cells types [121]. Moreover, few studies have paired miRNA profiling with analysis of mRNA from the same tissue. Regardless, a subset of miRNAs with changed expression after SE in at least two profiling studies have emerged. This list includes miRNAs -21, -30c, 125b, -132, -146a, -199a, and -375 among those upregulated and miRNAs -10b, -29a, -98, -181b,c, -374, -381, -450a, and -497 downregulated. Several appear in multiple studies including miR-132 and miR-146a which are consistently upregulated across models [114, 132, 133, 135]. In some cases, however, miRNAs have displayed bidirectional responses. For example, miR-21 has been reported to have protective effects against brain injury [136] was found down- [135] and upregulated [114].

In addition to expression profiling work, a number of studies focused on changes to individual miRNAs after SE. These include miRNAs -9, -34a, -125a, -134, -145, -150, and -155 [137–141]. In these cases, expression was invariably examined using real time quantitative PCR and Taqman-type assays. The insights from such studies remain rather limited in the absence of evidence of their upload to the RISC or binding to target mRNAs.

A number of studies have profiled miRNA changes in experimental epilepsy and a subset of miRNAs have been repeatedly found to be altered. Levels of miR-146a, which are consistently increased at later time points after SE [126, 127, 133, 137], was also reported to be upregulated in the hippocampus of epileptic rats [126]. Song and collaborators [142] reported that 18 miRNAs were upregulated and 5 miRNAs were downregulated in rat hippocampus 2 months after pilocarpine-induced SE. This is a consistent pattern, with upregulation of miRNAs being more common than downregulation in experimental epilepsy [132]. Others have found miR-146a increased in epilepsy [132, 138], and other miRNAs upregulated acutely after SE such as miR-132 and miR-134, remain elevated in established epilepsy [142]. miR-21 again features among the upregulated list in epilepsy [132] and has also been reported to be increased in models of prolonged seizures in immature rats [139].

A majority of expression profiling studies have used PCR- and microarray-based platforms to profile miRNA responses but a recent study used RNA sequencing. Unexpectedly, this resulted in rather few significantly differentially expressed miRNAs being detected in the hippocampus of epileptic rats (Increased: miR-455-3p, miR-345-3p, miR-423-3p, miR-54, miR-365-5p; Decreased: miR-296-5p) [143]. It is likely that small group sizes and low statistical power are responsible for the limited detection of differentially expressed miRNA in this study which should be carefully factored in any future RNAseq work.

Table 4.1 miRNA expression analyses in experimental and human epilepsy

Species	Model/age	Tissue/brain region	Platform	Profiled	Key findings	Ref.
Rat	Electrical stimulation of the angular bundle (adult)	Hippocampus (CA3)	Taqman assay	miR-146a	Upregulated Neuronal and glial expression	[126]
Rat	Pilocarpine-induced status epilepticus (adult)	Hippocampus (whole)	μParaflo™ microfluidic chip	349 miRNAs	23 regulated: 18 up, 5 down Increased miR-132, -134, -146a	[142]
Rat	Pilocarpine-induced status epilepticus (adult)	Hippocampus (whole)	Exiqon and Taqman arrays	miR-21	Upregulated Neurotrophin 3 identified as a target	[151]
Mouse	Intra-amygdala kainic acid-induced status epilepticus (adult)	Hippocampus (CA1, CA3)	Taqman assays	miR-134	Upregulated	[152]
Rat	Pilocarpine-induced status epilepticus in immature (P11/12) rats	Hippocampus (whole)	Invitrogen miRNA assay	miR-146a	Upregulated	[127]
Rat	Pilocarpine-induced status epilepticus (adult)	Hippocampus (whole)	Agilent array	>350 miRNA	24 (9 upregulated, 15 downregulated). Upregulated included miR-34a and miR-146a	[137]
Rat	Pilocarpine-induced status epilepticus (adult)	Hippocampus (whole)	Exiqon arrays	Genome-wide	36 regulated 33 increased 3 decreased Upregulated miR-146a	[132]

(continued)

Table 4.1 (continued)

Species	Model/age	Tissue/brain region	Platform	Profiled	Key findings	Ref.
Rat	Pilocarpine-induced status epilepticus (P25)	Hippocampus (whole)	Invitrogen miRNA assay	miR-21, miR-124, miR-132, miR-134	Upregulated	[139]
Rat	Pilocarpine-induced status epilepticus (P25)	Hippocampus (whole)	Takara miRNA assay	miR-155	Upregulated	[140]
Rat	Electrical stimulation of the amygdala	Hippocampus (dentate gyrus)	Exiqon arrays	Genome-wide	At the 30 day time point, 11 upregulated and 9 downregulated	[153]
					Upregulated miR-132 and -146a	
Rat	Electrical stimulation of the angular bundle	Hippocampus (isolated subfields and parahippocampal gyrus)	Exiqon arrays	Genome-wide	CA1: 5 upregulated	[133]
					DG: 37 upregulated, 5 downregulated	
					PHC: 22 upregulated, 7 downregulated	
					Upregulated miR-21, -132, -146a	
Rat	Electrical stimulation of the amygdala	Hippocampus (whole)	qRT-PCR after initially profiling post-status epilepticus by RNAseq (Illumina)	Genome-wide	5 upregulated (miR-874-3p, -20a-5p, -345-3p, -365-5p, -764-3p) and 1 downregulated (miR-99b-3p)	[154]
Mouse	Pilocarpine-induced status epilepticus (adult)	Whole brain	Agilent arrays	Genome-wide	31 miRNAs different in multi-drug resistant mice versus 21 different in anti-epileptic drug responders	[155]

(continued)

Table 4.1 (continued)

Species	Model/age	Tissue/brain region	Platform	Profiled	Key findings	Ref.
Rat	Pilocarpine-induced status epilepticus (adult)	Frontal cortex	Affymetrix	389 miRNAs	6 regulated	[156]
					3 upregulated	
					3 downregulated	
					Upregulation of miR-146a	
Rat	Electrical stimulation of the amygdala	Hippocampus (whole)	RNAseq (Illumina platform)	Genome-wide	25 differentially expressed	[157]
					14 upregulated	
					11 downregulated	
Rat	Pilocarpine-induced status epilepticus	Hippocampus (whole)	Taqman assays	miR-146a	Upregulated	[128]
Mouse	Pilocarpine-induced status epilepticus and electrical stimulation of the amygdala	Hippocampus (whole)	Exiqon array	Genome-wide	Pilocarpine model: 22 upregulated, 29 downregulated	[158]
					Electrical stimulation model: 41 upregulated, 16 downregulated	
Rat	Kainic acid-induced status epilepticus	Hippocampus (whole)	Exiqon miRNA q-RT-PCR	miR-129-5p	Increased	[159]
					Targets Kv1.1	
Human	Adults	Hippocampus ($n=20$ sclerotic and non-sclerotic)	Exiqon array	Genome-wide	165 different to control. Unique miRNA in HS vs. non-HS	[144]
					Unusual nuclear localization found for several miRNAs	
Human	Adults	Hippocampus ($n=3$)	Taqman low density arrays	380 miRNAs	Major downregulation of miRNA in samples with low Dicer expression	[113]
Human	Adults	Neocortex ($n=3$)	Taqman assays	miR-134	Higher expression	[152]

(continued)

Table 4.1 (continued)

Species	Model/age	Tissue/brain region	Platform	Profiled	Key findings	Ref.
Human	Children	Hippocampus ($n=5$)	Invitrogen miRNA assay	miR-146a	Higher expression	[127]
Human	Adults	Focal cortical dysplasia ($n=6$)	Taqman assays	miR-146a	Higher expression	[129]
Human	Children	Hippocampus ($n=5$)	Invitrogen miRNA assay	miR-21, miR-124, miR-132, miR-134	Higher expression	[139]
Human	Children	Hippocampus ($n=8$)	Takara miRNA assay	miR-155	Higher expression	[140]
Human	Children	Focal cortical dysplasia ($n=15$)	Agilent miRNA array	Genome-wide	10 regulated: 7 upregulated 3 downregulated Upregulated miR-21 and miR-155	[145]
Human	Adult	Temporal neocortex ($n=30$)	qRT-PCR	miR-132	Downregulated	[160]
Human	Adult	Dentate gyrus (with and without granule cell dispersion) ($n=14$)	Agilent array	~1200 miRNAs	12 regulated 6 upregulated 6 downregulated	[146]
Human	Adult	Hippocampus ($n=10$)	Exiqon arrays	Genome-wide	30 regulated 25 upregulated 5 downregulated	[161]
Human	Children	Cortex (tuberous sclerosis complex) ($n=5$)	Agilent array	>900 miRNAs	5 regulated 4 upregulated 1 downregulated Upregulation of miR-34a	[162]
Human	Adult	Hippocampus ($n=24$)	qRT-PCR	miR-199a-5p/b-5p	Downregulated	[163]

(continued)

Table 4.1 (continued)

Species	Model/age	Tissue/brain region	Platform	Profiled	Key findings	Ref.
Human	Adult	Hippocampus ($n=9$) with and without hippocampal sclerosis	Taqman arrays	~800 miRNAs	18 regulated	[150]
					12 differentially expressed in epilepsy regardless of pathology	
					6 unique to specific pathology	

Key: Studies are listed in chronological order of original online publication date. Search performed on Feb 25th, 2015 using search terms "epilepsy" and "microRNA" identified 85 studies. Table includes selected relevant experimental and human miRNA expression analyses. Notable miRNAs for which functional or other data are available are highlighted for some studies. *CA* cornu ammonis, *DG* dentate gyrus, *PHC* parahippocampal gyrus

miRNAs have also been profiled in resected human brain tissue from epileptic patients (Table 4.1). The first study, by Kan and collaborators, analyzed 20 mesial TLE patients' hippocampi (with and without sclerosis) and compared results to ten autopsy control tissue samples [144]. In this study, they reported similar numbers of up- and downregulated miRNAs with a total of 51 miRNAs differentially expressed by over twofold between epileptic and control groups. In contrast, the resected sclerotic human hippocampal samples analyzed by McKiernan and colleagues showed mainly downregulation of miRNAs. The collapse in miRNA production was attributed to the loss of Dicer in the tissue [113]. miRNA changes have also been reported in cortical dysplasia, another common cause of epilepsy [145]. Recently, 12 miRNAs were differentially expressed in sclerotic hippocampi samples from two groups of TLE patients with and without granule cell dispersion [146]. Among these miRNAs, miR-487a was highly expressed in an extended cohort of patients. ANTXR1, an adhesion molecule, was suggested as the possible target of miR-487a [146].

Molecular Mechanisms of miRNA Expression Control in Epilepsy

Almost nothing is known about what is driving the up- and downregulation of miRNAs in epilepsy. For some of the epilepsy-linked miRNAs such as miR-134, transcriptional mechanisms are known. Mef2 is known to be activated by neuronal activity and drives miR-134 expression in neurons [147]. However, in a majority of cases we do not yet know what promotes or represses miRNA expression in epilepsy. A recent study provided evidence that epigenetic mechanisms may be important regulators of miRNA expression in epilepsy. Epigenetic processes include

DNA methylation and histone modifications. Together these have powerful effects on the transcriptional state of chromatin, increasing or decreasing the degree of compaction and providing or restricting access to transcriptional machinery. Such epigenetic mechanisms are emerging as explanations of gene regulation in health and disease, including in epilepsy [148, 149].

Miller-Delaney and collaborators performed the first genome-wide analysis of DNA methylation in human epilepsy. The researchers found that 146 protein-coding genes exhibited altered DNA methylation in hippocampi of patients with TLE, with 81.5 % of methylation differences being hypermethylation [150]. They also investigated expression of miRNA in the same samples and correlated the degree of methylation with the corresponding expression (transcript level) of the miRNA. This identified a panel of 13 methylation-sensitive miRNA in the TLE samples whereby increased methylation was associated with lower expression of the miRNA. This included miR-27a, miR-193a-5p, and miR-876-3p. Differential methylation of long noncoding RNA in human TLE was also documented for the first time [150].

In summary, profiling analysis of experimental and human SE and epilepsy shows a select group of miRNAs undergo expression changes (Table 4.1). miRNA upregulation is the predominant response in both SE and epilepsy possibly contributing to reducing protein levels in the damaged brain. These studies provide rather limited insight into what the targets of these miRNAs are and whether they have causal roles in the pathogenesis of the epileptic state. Fortunately, a number of groups have undertaken functional studies on epilepsy-regulated miRNAs, results from which demonstrate miRNAs have a causal role in the epileptogenic process and epilepsy.

miRNA and Epilepsy: An Overview About Functional Studies

Researchers have several options available to manipulate miRNAs in vivo and verify whether they are relevant for epilepsy. The techniques mainly involve the use of genetic and oligonucleotide-based targeting of the miRNA [164, 165]. *Mimics* are used to increase miRNAs levels, while *antagomirs*, which bind to a specific miRNA inhibiting its function, can be used to decrease levels [165]. There are a number of transgenic and knockout mice available but these have not yet been studied in epilepsy [164, 165].

The first miRNA for which functional studies were performed in a seizure model was miR-132. Expression of miR-132 is consistently reported as upregulated after SE and epilepsy and Ago-2 pull-downs after SE in mice confirmed that miR-132 was uploaded to the RISC [114]. It is thought that higher levels of miR-132 may promote excitability. Indeed, it has been reported that miR-132 enhances the frequency and amplitude of excitatory currents in neurons [166], and increases dendritic length and branching by targeting p250GAP [167]. In vivo silencing of miR-132 through intracerebroventricular injection of *antagomirs* in mice was dem-

onstrated to reduce hippocampal damage caused by intra-amygdala KA-induced SE [114]. In more recent work, miR-132 inhibition was reported to reduce spontaneous seizures in rats and this was associated with reduced neuronal death [168]. However, treatment was initiated before SE therefore it remains unclear whether the reduction in spontaneous seizures was due to a modification of the initial insult or a truly disease-modifying effect on epilepsy.

The first miRNA for which a role in apoptosis was demonstrated was miR-34a [169]. It has been shown that miR-34a expression is controlled by p53, which is upregulated and contributes to neuronal death induced by seizures [170, 171]. Increased expression of miR-34a has been found in several reports and two studies have explored effects of antagomirs targeting miR-34a in seizure models. In one study silencing miR-34a reduced hippocampal damage after SE, but another study failed to detect such an effect. This may reflect model-specific contributions or other differences in the dosing or targeting of the miRNA [137, 141].

There has also been a single functional study on miR-184. This miRNA was identified as the most upregulated miRNA in the hippocampus after an episode of brief, non-harmful seizures, a model of epileptic preconditioning and a rich source of neuroprotective pathways [172]. McKiernan and colleagues showed that silencing miR-184 significantly increased seizure-induced neuronal death in two animal models of SE. This suggests that miR-184 may function to protect against seizure damage in the mouse [172].

A recent study revealed a strong link between miR-128 and convulsive behavior in mice. Mice with a conditional deletion of miR-128 were found to develop fatal epilepsy, which was fully prevented by treatment with an AED [122]. The mechanism was linked to increased excitatory transmission and mice lacking miR-128 displayed increased spine density. Complementing these findings, overexpression of miR-128 was able to suppress seizures triggered by kainic acid and effects were linked to the targets of miR-128 in the ERK2 network [122].

Another miRNA for which a comprehensive link to epilepsy has been demonstrated is for miR-134 (Fig. 4.1) [152]. miR-134 is a brain-enriched miRNA overexpressed after SE and in experimental and human epilepsy [152, 173]. Silencing miR-134 via *antagomirs*strongly reduced intra-amygdala KA-induced seizures with long-lasting protection [152] (Fig. 4.1). Recently, these protective effects were ratified using the pilocarpine model in mice [174]. In vitro antiseizure effects of targeting this miRNA have also been reported [175]. The mechanism of the antiseizure effect is uncertain but silencing miR-134 resulted in a slight decrease in hippocampal dendritic spine number in vivo which may reduce excitability [173]. This may relate to the target Limk-1, silencing of which obviated the protective effect of miR-134 in vitro [173]. Most importantly, intracerebroventricular injection of antagomirs targeting miR-134 reduced the occurrence of spontaneous seizures after SE in mice. This offers direct evidence for potential disease-modifying, anti-epileptogenic effects of targeting a miRNA (Fig. 4.1). Altogether, these data suggest that miR-134 may be a promising target to develop an antiepileptic therapy.

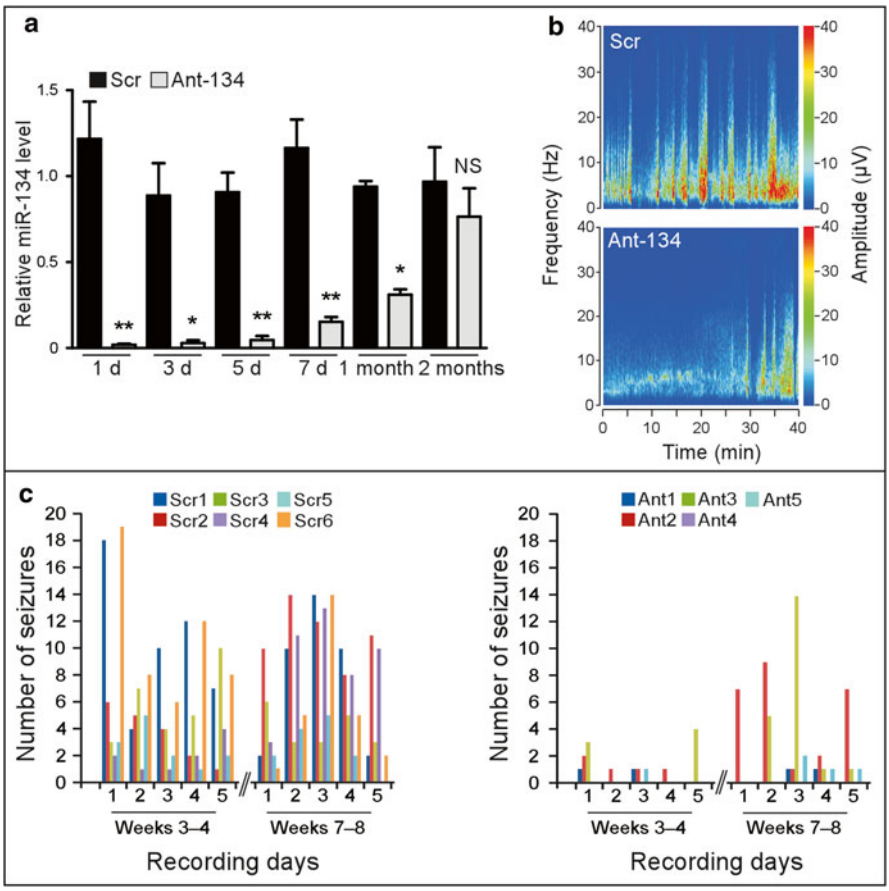

Fig. 4.1 *Antagomir* silencing of miR-134. (**a**) Analysis of miR-134 expression in the mouse hippocampus at various time points after a single intracerebroventricular injection of antagomirs targeting miR-134 showing long-lasting silencing in mouse hippocampus. (**b**) Representative EEG spectrograms show that *antagomir* silencing of miR-134 reduces seizure severity during *status epilepticus*. (**c**) Graphs show the number of generalized tonic clonic seizures each day for individual mice during two periods of 5 days of continuous video monitoring (1 and 2 months after kainic acid-induced SE) for scramble (Scr)- and antagomir (Ant)-injected mice. Ant-134 after *status epilepticus* reduces the number of epileptic seizures and protects against progressive TLE pathology (adapted and modified with permission from [152])

miRNA as Biomarkers of Epilepsy

An additional application of miRNAs in epilepsy relates to their potential use as biomarkers. Biomarkers are important diagnostic tools that reflect health or disease and which can be measured in a simple, ideally minimally invasive manner. There is a strong and urgent need for biomarkers of epilepsy. A biomarker or panel of biomarkers, found in biofluids such as blood would allow doctors to identify at-risk

patients after insults to the brain, predict seizure occurrence in patients with existing epilepsy, support prognosis and optimize or support discovery of therapies. There is a growing application of miRNAs as disease biomarkers using biofluid profiling [176, 177]. Biofluid miRNAs circulate in several forms, including enclosed in microparticles such as exosomes or complexed to Ago-2 and this seems to confer stability and a high reliability of detection. miRNAs are also ideal biomarkers because of their tissue-specific expression and roles in normal physiology and disease. There is a strong association between circulating and tissue levels although the sources of circulating miRNAs are still not well understood. It has been suggested that under healthy conditions miRNAs might be released from cells into the circulatory system. In disease or following injury, miRNAs derive from pathological processes within tissues [178, 179], for example, as by-products of necrotic or apoptotic cells [180]. There is also evidence that miRNAs are actively packaged and released in exosomes, functioning in a manner similar to hormones and cytokines in distant cell-to-cell communication [181].

A number of studies have investigated miRNA expression in blood after SE [134, 178], epileptogenesis, chronic epilepsy [178], stroke, intracerebral hemorrhage, and TBI [134, 179, 180]. They suggest there are unique miRNAs or groups of miRNAs which may reflect specific injuries to the brain or different phases of the epileptogenic process. These findings, whether validated in humans, might support diagnosis, prognosis, and patient treatment decisions not only in epilepsy, but also in other CNS disorders.

For complete review on circulating miRNAs as potential biomarkers in diagnosing CNS diseases the reader is referred elsewhere [121, 182–185].

Conclusion

There is now strong evidence supporting a role for miRNAs in the pathophysiology of epilepsy. Profiling work is increasingly providing a complete description of the expression levels and pathways under miRNA control in epilepsy. Concomitantly, researchers are establishing the in vivo functions of epilepsy-associated miRNAs and deploying RNA-based therapeutics as a novel approach to treatment. The use of miRNA inhibitors is already appearing in the medical literature. The first of these, miravirsen, was an antagomir blocking liver-expressed miR-122 which was found to be safe and effective in clinical trials of hepatitis C in humans [186]. Certainly, targeting miRNAs in CNS disorders will pose an additional challenge [187, 188]. This is because *antagomirs* are macromolecules that do not cross an intact blood brain barrier, which is essential for clinical use. Likewise, we need more data on the effects of *antagomirs* on the targets of the miRNAs and how to avoid off-targets effects. miRNAs also offer potential as biomarkers of epilepsy which may lead to novel diagnostics. In summary, miRNAs have emerged as important contributors to gene expression control in the brain. They offer a perhaps unique combination of therapeutic opportunities and co-developed molecular diagnostics for epilepsy.

Acknowledgements The authors apologize to those authors whose relevant work was not cited here. microRNA research in the authors' group is currently supported by grants from Health Research Board (HRA-POR-2013-325), European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 602130, Science Foundation Ireland (12/RC/2272, 12/COEN/18, 13/IA/1891, 14/ADV/RC2721), and Irish Research Council (GOIPD/2014/566).

References

1. Loscher W, Brandt C. Prevention or modification of epileptogenesis after brain insults: experimental approaches and translational research. Pharmacol Rev. 2010;62:668–700.
2. Vezzani A, French J, Bartfai T, Baram TZ. The role of inflammation in epilepsy. Nat Rev Neurol. 2011;7:31–40.
3. Fisher RS, Acevedo C, Arzimanoglou A, Bogacz A, Cross JH, Elger CE, et al. ILAE official report: a practical clinical definition of epilepsy. Epilepsia. 2014;55:475–82.
4. Engel Jr J. Classification of epileptic disorders. Epilepsia. 2001;42:316.
5. Thurman DJ, Beghi E, Begley CE, Berg AT, Buchhalter JR, Ding D, et al. Standards for epidemiologic studies and surveillance of epilepsy. Epilepsia. 2011;52 Suppl 7:2–26.
6. Fisher RS, van Emde BW, Blume W, Elger C, Genton P, Lee P, et al. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia. 2005;46:470–2.
7. Proposal for revised clinical and electroencephalographic classification of epileptic seizures. From the commission on classification and terminology of the international league against epilepsy. Epilepsia. 1981;22:489–501.
8. Jenssen S, Gracely EJ, Sperling MR. How long do most seizures last? A systematic comparison of seizures recorded in the epilepsy monitoring unit. Epilepsia. 2006;47:1499–503.
9. Shinnar S, Berg AT, Moshe SL, Shinnar R. How long do new-onset seizures in children last? Ann Neurol. 2001;49:659–64.
10. Al-Mufti F, Claassen J. Neurocritical care: status epilepticus review. Crit Care Clin. 2014;30:751–64.
11. Meldrum BS, Brierley JB. Prolonged epileptic seizures in primates. Ischemic cell change and its relation to ictal physiological events. Arch Neurol. 1973;28:10–7.
12. Moshe SL, Perucca E, Ryvlin P, Tomson T. Epilepsy: new advances. Lancet. 2015;385(9971):884–98.
13. Thomas RH, Berkovic SF. The hidden genetics of epilepsy-a clinically important new paradigm. Nat Rev Neurol. 2014;10:283–92.
14. Ngugi AK, Bottomley C, Kleinschmidt I, Sander JW, Newton CR. Estimation of the burden of active and life-time epilepsy: a meta-analytic approach. Epilepsia. 2010;51:883–90.
15. Savage N. Epidemiology: the complexities of epilepsy. Nature. 2014;511:S2–3.
16. Hauser WA, Annegers JF, Kurland LT. Incidence of epilepsy and unprovoked seizures in Rochester, Minnesota: 1935–1984. Epilepsia. 1993;34:453–68.
17. Kotsopoulos IA, van Merode T, Kessels FG, de Krom MC, Knottnerus JA. Systematic review and meta-analysis of incidence studies of epilepsy and unprovoked seizures. Epilepsia. 2002;43:1402–9.
18. Christensen J, Kjeldsen MJ, Andersen H, Friis ML, Sidenius P. Gender differences in epilepsy. Epilepsia. 2005;46:956–60.
19. McHugh JC, Delanty N. Epidemiology and classification of epilepsy: gender comparisons. Int Rev Neurobiol. 2008;83:11–26.
20. Savic I. Sex differences in human epilepsy. Exp Neurol. 2014;259:38–43.
21. Benbadis S. The differential diagnosis of epilepsy: a critical review. Epilepsy Behav. 2009;15:15–21.

22. Rogawski MA. Diverse mechanisms of antiepileptic drugs in the development pipeline. Epilepsy Res. 2006;69:273–94.
23. Rogawski MA, Hanada T. Preclinical pharmacology of perampanel, a selective non-competitive AMPA receptor antagonist. Acta Neurol Scand Suppl. 2013:19–24.
24. Perucca E, French J, Bialer M. Development of new antiepileptic drugs: challenges, incentives, and recent advances. Lancet Neurol. 2007;6:793–804.
25. Fisher RS. Therapeutic devices for epilepsy. Ann Neurol. 2012;71:157–68.
26. Pitkänen A, Schwartzkroin PA, Moshé SL. Models of seizures and epilepsy. Oxford: Elsevier Academic Press; 2006. 712 p.
27. Coppola A, Moshe SL. Animal models. Handb Clin Neurol. 2012;107:63–98.
28. Loscher W. Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. Seizure. 2011;20:359–68.
29. Simonato M, Brooks-Kayal AR, Engel Jr J, Galanopoulou AS, Jensen FE, Moshe SL, et al. The challenge and promise of anti-epileptic therapy development in animal models. Lancet Neurol. 2014;13:949–60.
30. Engel Jr J, International League Against Epilepsy. A proposed diagnostic scheme for people with epileptic seizures and with epilepsy: report of the ILAE Task Force on Classification and Terminology. Epilepsia. 2001;42:796–803.
31. McGeer PL, McGeer EG. Intracerebral injections of kainic acid and tetanus toxin: possible models for the signs of chorea and dystonia. Adv Neurol. 1978;21:331–8.
32. Coyle JT. Kainic acid: insights into excitatory mechanisms causing selective neuronal degeneration. Ciba Found Symp. 1987;126:186–203.
33. Miller LP, Johnson AE, Gelhard RE, Insel TR. The ontogeny of excitatory amino acid receptors in the rat forebrain—II. Kainic acid receptors. Neuroscience. 1990;35:45–51.
34. Schwarcz R, Zaczek R, Coyle JT. Microinjection of kainic acid into the rat hippocampus. Eur J Pharmacol. 1978;50:209–20.
35. Rondouin G, Bockaert J, Lerner-Natoli M. L-nitroarginine, an inhibitor of NO synthase, dramatically worsens limbic epilepsy in rats. Neuroreport. 1993;4:1187–90.
36. Ben-Ari Y. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neuroscience. 1985;14:375–403.
37. Cavalheiro EA, Riche DA, Le Gal La Salle G. Long-term effects of intrahippocampal kainic acid injection in rats: a method for inducing spontaneous recurrent seizures. Electroencephalogr Clin Neurophysiol. 1982;53:581–9.
38. Stafstrom CE, Thompson JL, Holmes GL. Kainic acid seizures in the developing brain: status epilepticus and spontaneous recurrent seizures. Brain Res Dev Brain Res. 1992;65:227–36.
39. Giorgi FS, Malhotra S, Hasson H, Veliskova J, Rosenbaum DM, Moshe SL. Effects of status epilepticus early in life on susceptibility to ischemic injury in adulthood. Epilepsia. 2005;46:490–8.
40. Velisek L. Models of chemically-induced acute seizures. In: Pitkanen A, Schwartzkroin PA, Moshé S, editors. Models of seizure and epilepsy. Elsevier Academic Press: Oxford; 2006. p. 127–52.
41. Kuhar M, Yamamura HI. Localization of cholinergic muscarinic receptors in rat brain by light microscopic radioautography. Brain Res. 1976;110:229–43.
42. Fabene PF, Navarro Mora G, Martinello M, Rossi B, Merigo F, Ottoboni L, et al. A role for leukocyte-endothelial adhesion mechanisms in epilepsy. Nat Med. 2008;14:1377–83.
43. Oby E, Janigro D. The blood–brain barrier and epilepsy. Epilepsia. 2006;47:1761–74.
44. Marchi N, Angelov L, Masaryk T, Fazio V, Granata T, Hernandez N, et al. Seizure-promoting effect of blood–brain barrier disruption. Epilepsia. 2007;48:732–42.
45. Goddard GV, McIntyre DC, Leech CK. A permanent change in brain function resulting from daily electrical stimulation. Exp Neurol. 1969;25:295–330.
46. McNamara JO, Byrne MC, Dasheiff RM, Fitz JG. The kindling model of epilepsy: a review. Prog Neurobiol. 1980;15:139–59.
47. Morrell F. Secondary epileptogenic lesions. Epilepsia. 1960;1:538–60.

48. Bertram E. The relevance of kindling for human epilepsy. Epilepsia. 2007;48 Suppl 2:65–74.
49. Chang BS, Lowenstein DH. Epilepsy. N Engl J Med. 2003;349:1257–66.
50. Hanna MG. Genetic neurological channelopathies. Nat Clin Pract Neurol. 2006;2:252–63.
51. Maljevic S, Lerche H. Potassium channel genes and benign familial neonatal epilepsy. Prog Brain Res. 2014;213:17–53.
52. Epi4K Consortium, Epilepsy Phenome/Genome Project, Allen AS, Berkovic SF, Cossette P, Delanty N, et al. De novo mutations in epileptic encephalopathies. Nature. 2013;501:217–21.
53. Gomez-Herreros F, Schuurs-Hoeijmakers JH, McCormack M, Greally MT, Rulten S, Romero-Granados R, et al. TDP2 protects transcription from abortive topoisomerase activity and is required for normal neural function. Nat Genet. 2014;46:516–21.
54. Jin P, Alisch RS, Warren ST. RNA and microRNAs in fragile X mental retardation. Nat Cell Biol. 2004;6:1048–53.
55. Piccione M, Vecchio D, Cavani S, Malacarne M, Pierluigi M, Corsello G. The first case of myoclonic epilepsy in a child with a de novo 22q11.2 microduplication. Am J Med Genet A. 2011;155A:3054–9.
56. Cheung EN, George SR, Andrade DM, Chow EW, Silversides CK, Bassett AS. Neonatal hypocalcemia, neonatal seizures, and intellectual disability in 22q11.2 deletion syndrome. Genet Med. 2014;16:40–4.
57. Wieser HG, ILAE Commission on Neurosurgery of Epilepsy. ILAE commission report. Mesial temporal lobe epilepsy with hippocampal sclerosis. Epilepsia. 2004;45:695–714.
58. Thom M, Blumcke I, Aronica E. Long-term epilepsy-associated tumors. Brain Pathol. 2012;22:350–79.
59. Thom M. Review: hippocampal sclerosis in epilepsy: a neuropathology review. Neuropathol Appl Neurobiol. 2014;40:520–43.
60. Okamoto OK, Janjoppi L, Bonone FM, Pansani AP, da Silva AV, Scorza FA, et al. Whole transcriptome analysis of the hippocampus: toward a molecular portrait of epileptogenesis. BMC Genomics. 2010;11:230.
61. Kharatishvili I, Pitkanen A. Association of the severity of cortical damage with the occurrence of spontaneous seizures and hyperexcitability in an animal model of posttraumatic epilepsy. Epilepsy Res. 2010;90:47–59.
62. Ebert U, Brandt C, Loscher W. Delayed sclerosis, neuroprotection, and limbic epileptogenesis after status epilepticus in the rat. Epilepsia. 2002;43 Suppl 5:86–95.
63. Andre V, Ferrandon A, Marescaux C, Nehlig A. Electroshocks delay seizures and subsequent epileptogenesis but do not prevent neuronal damage in the lithium-pilocarpine model of epilepsy. Epilepsy Res. 2000;42:7–22.
64. Sloviter RS, Bumanglag AV. Defining "epileptogenesis" and identifying "antiepileptogenic targets" in animal models of acquired temporal lobe epilepsy is not as simple as it might seem. Neuropharmacology. 2013;69:3–15.
65. Vezzani A, Aronica E, Mazarati A, Pittman QJ. Epilepsy and brain inflammation. Exp Neurol. 2013;244:11–21.
66. Engel T, Murphy BM, Hatazaki S, Jimenez-Mateos EM, Concannon CG, Woods I, et al. Reduced hippocampal damage and epileptic seizures after status epilepticus in mice lacking proapoptotic Puma. FASEB J. 2010;24:853–61.
67. Engel T, Sanz-Rodgriguez A, Jimenez-Mateos EM, Concannon CG, Jimenez-Pacheco A, Moran C, et al. CHOP regulates the p53-MDM2 axis and is required for neuronal survival after seizures. Brain. 2013;136:577–92.
68. Vezzani A, Granata T. Brain inflammation in epilepsy: experimental and clinical evidence. Epilepsia. 2005;46:1724–43.
69. Pitkanen A, Sutula TP. Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. Lancet Neurol. 2002;1:173–81.
70. Aronica E, Gorter JA. Gene expression profile in temporal lobe epilepsy. Neuroscientist. 2007;13:100–8.

71. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. Trends Neurosci. 1996;19:312–8.
72. Choi J, Nordli Jr DR, Alden TD, DiPatri Jr A, Laux L, Kelley K, et al. Cellular injury and neuroinflammation in children with chronic intractable epilepsy. J Neuroinflammation. 2009;6:38.
73. Jorgensen MB, Finsen BR, Jensen MB, Castellano B, Diemer NH, Zimmer J. Microglial and astroglial reactions to ischemic and kainic acid-induced lesions of the adult rat hippocampus. Exp Neurol. 1993;120:70–88.
74. Borges K, Gearing M, McDermott DL, Smith AB, Almonte AG, Wainer BH, et al. Neuronal and glial pathological changes during epileptogenesis in the mouse pilocarpine model. Exp Neurol. 2003;182:21–34.
75. da Fonseca AC, Matias D, Garcia C, Amaral R, Geraldo LH, Freitas C, et al. The impact of microglial activation on blood–brain barrier in brain diseases. Front Cell Neurosci. 2014;8:362.
76. Obermeier B, Daneman R, Ransohoff RM. Development, maintenance and disruption of the blood–brain barrier. Nat Med. 2013;19:1584–96.
77. Varvel NH, Jiang J, Dingledine R. Candidate drug targets for prevention or modification of epilepsy. Annu Rev Pharmacol Toxicol. 2015;55:229–47.
78. Ravizza T, Gagliardi B, Noe F, Boer K, Aronica E, Vezzani A. Innate and adaptive immunity during epileptogenesis and spontaneous seizures: evidence from experimental models and human temporal lobe epilepsy. Neurobiol Dis. 2008;29:142–60.
79. Oliveira MS, Furian AF, Royes LF, Fighera MR, Fiorenza NG, Castelli M, et al. Cyclooxygenase-2/PGE2 pathway facilitates pentylenetetrazol-induced seizures. Epilepsy Res. 2008;79:14–21.
80. Rojas A, Jiang J, Ganesh T, Yang MS, Lelutiu N, Gueorguieva P, et al. Cyclooxygenase-2 in epilepsy. Epilepsia. 2014;55:17–25.
81. Maroso M, Balosso S, Ravizza T, Liu J, Aronica E, Iyer AM, et al. Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat Med. 2010;16:413–9.
82. Roth TL, Nayak D, Atanasijevic T, Koretsky AP, Latour LL, McGavern DB. Transcranial amelioration of inflammation and cell death after brain injury. Nature. 2014;505:223–8.
83. Fieschi C, Lenzi GL, Zanette E, Orzi F, Passero S. Effects on EEG of the osmotic opening of the blood–brain barrier in rats. Life Sci. 1980;27:239–43.
84. Uva L, Librizzi L, Marchi N, Noe F, Bongiovanni R, Vezzani A, et al. Acute induction of epileptiform discharges by pilocarpine in the in vitro isolated guinea-pig brain requires enhancement of blood–brain barrier permeability. Neuroscience. 2008;151:303–12.
85. van Vliet EA, da Costa AS, Redeker S, van Schaik R, Aronica E, Gorter JA. Blood–brain barrier leakage may lead to progression of temporal lobe epilepsy. Brain. 2007;130:521–34.
86. Ivens S, Kaufer D, Flores LP, Bechmann I, Zumsteg D, Tomkins O, et al. TGF-beta receptor-mediated albumin uptake into astrocytes is involved in neocortical epileptogenesis. Brain. 2007;130:535–47.
87. David Y, Cacheaux LP, Ivens S, Lapilover E, Heinemann U, Kaufer D, et al. Astrocytic dysfunction in epileptogenesis: consequence of altered potassium and glutamate homeostasis? J Neurosci. 2009;29:10588–99.
88. Loscher W, Potschka H. Drug resistance in brain diseases and the role of drug efflux transporters. Nat Rev Neurosci. 2005;6:591–602.
89. Tauck DL, Nadler JV. Evidence of functional mossy fiber sprouting in hippocampal formation of kainic acid-treated rats. J Neurosci. 1985;5:1016–22.
90. Cavazos JE, Golarai G, Sutula TP. Mossy fiber synaptic reorganization induced by kindling: time course of development, progression, and permanence. J Neurosci. 1991;11:2795–803.
91. Nadler JV, Perry BW, Gentry C, Cotman CW. Degeneration of hippocampal CA3 pyramidal cells induced by intraventricular kainic acid. J Comp Neurol. 1980;192:333–59.
92. Sutula T, Cascino G, Cavazos J, Parada I, Ramirez L. Mossy fiber synaptic reorganization in the epileptic human temporal lobe. Ann Neurol. 1989;26:321–30.

93. Cronin J, Dudek FE. Chronic seizures and collateral sprouting of dentate mossy fibers after kainic acid treatment in rats. Brain Res. 1988;474:181–4.
94. Masukawa LM, Uruno K, Sperling M, O'Connor MJ, Burdette LJ. The functional relationship between antidromically evoked field responses of the dentate gyrus and mossy fiber reorganization in temporal lobe epileptic patients. Brain Res. 1992;579:119–27.
95. Heng K, Haney MM, Buckmaster PS. High-dose rapamycin blocks mossy fiber sprouting but not seizures in a mouse model of temporal lobe epilepsy. Epilepsia. 2013;54:1535–41.
96. Schirle NT, MacRae IJ. The crystal structure of human Argonaute2. Science. 2012;336:1037–40.
97. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. Nat Rev Cancer. 2006;6:259–69.
98. Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. Nature. 2008;455:58–63.
99. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. Curr Biol. 2002;12:735–9.
100. Krichevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS. A microRNA array reveals extensive regulation of microRNAs during brain development. RNA. 2003;9:1274–81.
101. Jimenez-Mateos EM, Henshall DC. Epilepsy and microRNA. Neuroscience. 2013;238:218–29.
102. O'Carroll D, Schaefer A. General principals of miRNA biogenesis and regulation in the brain. Neuropsychopharmacology. 2013;38:39–54.
103. Siegel C, Li J, Liu F, Benashski SE, McCullough LD. miR-23a regulation of X-linked inhibitor of apoptosis (XIAP) contributes to sex differences in the response to cerebral ischemia. Proc Natl Acad Sci U S A. 2011;108:11662–7.
104. Lugli G, Torvik VI, Larson J, Smalheiser NR. Expression of microRNAs and their precursors in synaptic fractions of adult mouse forebrain. J Neurochem. 2008;106:650–61.
105. Davis TH, Cuellar TL, Koch SM, Barker AJ, Harfe BD, McManus MT, et al. Conditional loss of Dicer disrupts cellular and tissue morphogenesis in the cortex and hippocampus. J Neurosci. 2008;28:4322–30.
106. De Pietri TD, Pulvers JN, Haffner C, Murchison EP, Hannon GJ, Huttner WB. miRNAs are essential for survival and differentiation of newborn neurons but not for expansion of neural progenitors during early neurogenesis in the mouse embryonic neocortex. Development. 2008;135:3911–21.
107. Babiarz JE, Hsu R, Melton C, Thomas M, Ullian EM, Blelloch R. A role for noncanonical microRNAs in the mammalian brain revealed by phenotypic differences in Dgcr8 versus Dicer1 knockouts and small RNA sequencing. RNA. 2011;17:1489–501.
108. Tao J, Wu H, Lin Q, Wei W, Lu XH, Cantle JP, et al. Deletion of astroglial Dicer causes non-cell-autonomous neuronal dysfunction and degeneration. J Neurosci. 2011;31:8306–19.
109. Knuckles P, Vogt MA, Lugert S, Milo M, Chong MM, Hautbergue GM, et al. Drosha regulates neurogenesis by controlling neurogenin 2 expression independent of microRNAs. Nat Neurosci. 2012;15:962–9.
110. Hsu R, Schofield CM, Dela Cruz CG, Jones-Davis DM, Blelloch R, Ullian EM. Loss of microRNAs in pyramidal neurons leads to specific changes in inhibitory synaptic transmission in the prefrontal cortex. Mol Cell Neurosci. 2012;50:283–92.
111. Morita S, Horii T, Kimura M, Goto Y, Ochiya T, Hatada I. One Argonaute family member, Eif2c2 (Ago2), is essential for development and appears not to be involved in DNA methylation. Genomics. 2007;89:687–96.
112. Kawase-Koga Y, Otaegi G, Sun T. Different timings of Dicer deletion affect neurogenesis and gliogenesis in the developing mouse central nervous system. Dev Dyn. 2009;238:2800–12.
113. McKiernan RC, Jimenez-Mateos EM, Bray I, Engel T, Brennan GP, Sano T, et al. Reduced mature microRNA levels in association with dicer loss in human temporal lobe epilepsy with hippocampal sclerosis. PLoS One. 2012;7, e35921.
114. Jimenez-Mateos EM, Bray I, Sanz-Rodriguez A, Engel T, McKiernan RC, Mouri G, et al. miRNA Expression profile after status epilepticus and hippocampal neuroprotection by targeting miR-132. Am J Pathol. 2011;179:2519–32.

115. Nakagawa A, Shi Y, Kage-Nakadai E, Mitani S, Xue D. Caspase-dependent conversion of Dicer ribonuclease into a death-promoting deoxyribonuclease. Science. 2010;328:327–34.
116. Schindler CK, Pearson EG, Bonner HP, So NK, Simon RP, Prehn JH, et al. Caspase-3 cleavage and nuclear localization of caspase-activated DNase in human temporal lobe epilepsy. J Cereb Blood Flow Metab. 2006;26:583–9.
117. Bicker S, Lackinger M, Weiss K, Schratt G. MicroRNA-132, -134, and -138: a microRNA troika rules in neuronal dendrites. Cell Mol Life Sci. 2014;71:3987–4005.
118. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19:92–105.
119. Pitkanen A, Lukasiuk K. Mechanisms of epileptogenesis and potential treatment targets. Lancet Neurol. 2011;10:173–86.
120. Dogini DB, Avansini SH, Vieira AS, Lopes-Cendes I. MicroRNA regulation and dysregulation in epilepsy. Front Cell Neurosci. 2013;7:172.
121. Henshall DC. MicroRNAs in the pathophysiology and treatment of status epilepticus. Front Mol Neurosci. 2013;6:37.
122. Tan CL, Plotkin JL, Veno MT, von Schimmelmann M, Feinberg P, Mann S, et al. MicroRNA-128 governs neuronal excitability and motor behavior in mice. Science. 2013;342:1254–8.
123. Letellier M, Elramah S, Mondin M, Soula A, Penn A, Choquet D, et al. miR-92a regulates expression of synaptic GluA1-containing AMPA receptors during homeostatic scaling. Nat Neurosci. 2014;17:1040–2.
124. Siegel G, Saba R, Schratt G. microRNAs in neurons: manifold regulatory roles at the synapse. Curr Opin Genet Dev. 2011;21:491–7.
125. Sheedy FJ, O'Neill LA. Adding fuel to fire: microRNAs as a new class of mediators of inflammation. Ann Rheum Dis. 2008;67 Suppl 3:iii50–5.
126. Aronica E, Fluiter K, Iyer A, Zurolo E, Vreijling J, van Vliet EA, et al. Expression pattern of miR-146a, an inflammation-associated microRNA, in experimental and human temporal lobe epilepsy. Eur J Neurosci. 2010;31:1100–7.
127. Omran A, Peng J, Zhang C, Xiang QL, Xue J, Gan N, et al. Interleukin-1beta and microRNA-146a in an immature rat model and children with mesial temporal lobe epilepsy. Epilepsia. 2012;53:1215–24.
128. de Araujo MA, Marques TE, Taniele-Silva J, Souza FM, de Andrade TG, Garcia-Cairasco N, et al. Identification of endogenous reference genes for the analysis of microRNA expression in the hippocampus of the pilocarpine-induced model of mesial temporal lobe epilepsy. PLoS One. 2014;9, e100529.
129. Iyer A, Zurolo E, Prabowo A, Fluiter K, Spliet WG, van Rijen PC, et al. MicroRNA-146a: a key regulator of astrocyte-mediated inflammatory response. PLoS One. 2012;7, e44789.
130. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A. 2006;103:12481–6.
131. Henshall DC. MicroRNAs in the pathophysiology and treatment of status epilepticus. Front Mol Neurosci. 2013;6:1–11.
132. Risbud RM, Porter BE. Changes in microRNA expression in the whole hippocampus and hippocampal synaptoneurosome fraction following pilocarpine induced status epilepticus. PLoS One. 2013;8, e53464.
133. Gorter JA, Iyer A, White I, Colzi A, van Vliet EA, Sisodiya S, et al. Hippocampal subregion-specific microRNA expression during epileptogenesis in experimental temporal lobe epilepsy. Neurobiol Dis. 2014;62:508–20.
134. Liu DZ, Tian Y, Ander BP, Xu H, Stamova BS, Zhan X, et al. Brain and blood microRNA expression profiling of ischemic stroke, intracerebral hemorrhage, and kainate seizures. J Cereb Blood Flow Metab. 2010;30:92–101.
135. Hu K, Zhang C, Long L, Long X, Feng L, Li Y, et al. Expression profile of microRNAs in rat hippocampus following lithium-pilocarpine-induced status epilepticus. Neurosci Lett. 2011;488:252–7.

136. Ge XT, Lei P, Wang HC, Zhang AL, Han ZL, Chen X, et al. miR-21 improves the neurological outcome after traumatic brain injury in rats. Sci Rep. 2014;4:6718.
137. Hu K, Xie YY, Zhang C, Ouyang DS, Long HY, Sun DN, et al. MicroRNA expression profile of the hippocampus in a rat model of temporal lobe epilepsy and miR-34a-targeted neuroprotection against hippocampal neurone cell apoptosis post-status epilepticus. BMC Neurosci. 2012;13:115.
138. Pichardo-Casas I, Goff LA, Swerdel MR, Athie A, Davila J, Ramos-Brossier M, et al. Expression profiling of synaptic microRNAs from the adult rat brain identifies regional differences and seizure-induced dynamic modulation. Brain Res. 2012;1436:20–33.
139. Peng J, Omran A, Ashhab MU, Kong H, Gan N, He F, et al. Expression patterns of miR-124, miR-134, miR-132, and miR-21 in an immature rat model and children with mesial temporal lobe epilepsy. J Mol Neurosci. 2013;50:291–7.
140. Ashhab MU, Omran A, Kong H, Gan N, He F, Peng J, et al. Expressions of tumor necrosis factor alpha and microRNA-155 in immature rat model of status epilepticus and children with mesial temporal lobe epilepsy. J Mol Neurosci. 2013;51:950–8.
141. Sano T, Reynolds JP, Jimenez-Mateos EM, Matsushima S, Taki W, Henshall DC. MicroRNA-34a upregulation during seizure-induced neuronal death. Cell Death Dis. 2012;3, e287.
142. Song YJ, Tian XB, Zhang S, Zhang YX, Li X, Li D, et al. Temporal lobe epilepsy induces differential expression of hippocampal miRNAs including let-7e and miR-23a/b. Brain Res. 2011;1387:134–40.
143. Li MM, Jiang T, Sun Z, Zhang Q, Tan CC, Yu JT, et al. Genome-wide microRNA expression profiles in hippocampus of rats with chronic temporal lobe epilepsy. Sci Rep. 2014;4:4734.
144. Kan AA, van Erp S, Derijck AA, de Wit M, Hessel EV, O'Duibhir E, et al. Genome-wide microRNA profiling of human temporal lobe epilepsy identifies modulators of the immune response. Cell Mol Life Sci. 2012;69:3127–45.
145. Lee JY, Park AK, Lee ES, Park WY, Park SH, Choi JW, et al. miRNA expression analysis in cortical dysplasia: regulation of mTOR and LIS1 pathway. Epilepsy Res. 2014;108:433–41.
146. Zucchini S, Marucci G, Paradiso B, Lanza G, Roncon P, Cifelli P, et al. Identification of miRNAs differentially expressed in human epilepsy with or without granule cell pathology. PLoS One. 2014;9, e105521.
147. Fiore R, Khudayberdiev S, Christensen M, Siegel G, Flavell SW, Kim TK, et al. Mef2-mediated transcription of the miR379-410 cluster regulates activity-dependent dendritogenesis by fine-tuning Pumilio2 protein levels. EMBO J. 2009;28:697–710.
148. Boison D, Sandau US, Ruskin DN, Kawamura Jr M, Masino SA. Homeostatic control of brain function—new approaches to understand epileptogenesis. Front Cell Neurosci. 2013;7:109.
149. Roopra A, Dingledine R, Hsieh J. Epigenetics and epilepsy. Epilepsia. 2012;53 Suppl 9:2–10.
150. Miller-Delaney SF, Bryan K, Das S, McKiernan RC, Bray IM, Reynolds JP, et al. Differential DNA methylation profiles of coding and non-coding genes define hippocampal sclerosis in human temporal lobe epilepsy. Brain. 2015;138:616–31.
151. Risbud RM, Lee C, Porter BE. Neurotrophin-3 mRNA a putative target of miR21 following status epilepticus. Brain Res. 2011;1424:53–9.
152. Jimenez-Mateos EM, Engel T, Merino-Serrais P, McKiernan RC, Tanaka K, Mouri G, et al. Silencing microRNA-134 produces neuroprotective and prolonged seizure-suppressive effects. Nat Med. 2012;18:1087–94.
153. Bot AM, Debski KJ, Lukasiuk K. Alterations in miRNA levels in the dentate gyrus in epileptic rats. PLoS One. 2013;8, e76051.
154. Sun Z, Yu JT, Jiang T, Li MM, Tan L, Zhang Q, et al. Genome-wide microRNA profiling of rat hippocampus after status epilepticus induced by amygdala stimulation identifies modulators of neuronal apoptosis. PLoS One. 2013;8, e78375.
155. Moon J, Lee ST, Choi J, Jung KH, Yang H, Khalid A, et al. Unique behavioral characteristics and microRNA signatures in a drug resistant epilepsy model. PLoS One. 2014;9, e85617.

156. Matos G, Scorza FA, Mazzotti DR, Guindalini C, Cavalheiro EA, Tufik S, et al. The effects of sleep deprivation on microRNA expression in rats submitted to pilocarpine-induced status epilepticus. Prog Neuropsychopharmacol Biol Psychiatry. 2014;51:159–65.
157. Li MM, Jiang T, Sun Z, Zhang Q, Tan CC, Yu JT, Tan L. Genome-wide microRNA expression profiles in hippocampus of rats with chronic temporal lobe epilepsy. Sci Rep. 2014; 4, 4734.
158. Kretschmann A, Danis B, Andonovic L, Abnaof K, van Rikxoort M, Siegel F, et al. Different microRNA profiles in chronic epilepsy versus acute seizure mouse models. J Mol Neurosci. 2015;55:466–79.
159. Sosanya NM, Brager DH, Wolfe S, Niere F, Raab-Graham KF. Rapamycin reveals an mTOR-independent repression of Kv1.1 expression during epileptogenesis. Neurobiol Dis. 2015;73:96–105.
160. Guo J, Wang H, Wang Q, Chen Y, Chen S. Expression of p-CREB and activity-dependent miR-132 in temporal lobe epilepsy. Int J Clin Exp Med. 2014;7:1297–306.
161. Kaalund SS, Veno MT, Bak M, Moller RS, Laursen H, Madsen F, et al. Aberrant expression of miR-218 and miR-204 in human mesial temporal lobe epilepsy and hippocampal sclerosis-convergence on axonal guidance. Epilepsia. 2014;55:2017–27.
162. Dombkowski AA, Batista CE, Cukovic D, Carruthers NJ, Ranganathan R, Shukla U, et al. Cortical tubers: windows into dysregulation of epilepsy risk and synaptic signaling genes by microRNAs. Cereb Cortex. 2014.
163. Jiang G, Zhou R, He X, Shi Z, Huang M, Yu J, et al. Expression levels of microRNA-199 and hypoxia-inducible factor-1 alpha in brain tissue of patients with intractable epilepsy. Int J Neurosci. 2014:1–29.
164. Brown BD, Naldini L. Exploiting and antagonizing microRNA regulation for therapeutic and experimental applications. Nat Rev Genet. 2009;10:578–85.
165. Bhalala OG, Srikanth M, Kessler JA. The emerging roles of microRNAs in CNS injuries. Nat Rev Neurol. 2013;9:328–39.
166. Edbauer D, Neilson JR, Foster KA, Wang CF, Seeburg DP, Batterton MN, et al. Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. Neuron. 2010;65:373–84.
167. Wayman GA, Davare M, Ando H, Fortin D, Varlamova O, Cheng HY, et al. An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP. Proc Natl Acad Sci U S A. 2008;105:9093–8.
168. Huang Y, Guo J, Wang Q, Chen Y. MicroRNA-132 silencing decreases the spontaneous recurrent seizures. Int J Clin Exp Med. 2014;7:1639–49.
169. Welch C, Chen Y, Stallings RL. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. Oncogene. 2007;26:5017–22.
170. Morrison RS, Wenzel HJ, Kinoshita Y, Robbins CA, Donehower LA, Schwartzkroin PA. Loss of the p53 tumor suppressor gene protects neurons from kainate—induced cell death. J Neurosci. 1996;16:1337–45.
171. Engel T, Tanaka K, Jimenez-Mateos EM, Caballero-Caballero A, Prehn JH, Henshall DC. Loss of p53 results in protracted electrographic seizures and development of an aggravated epileptic phenotype following status epilepticus. Cell Death Dis. 2010;1, e79.
172. McKiernan RC, Jimenez-Mateos EM, Sano T, Bray I, Stallings RL, Simon RP, et al. Expression profiling the microRNA response to epileptic preconditioning identifies miR-184 as a modulator of seizure-induced neuronal death. Exp Neurol. 2012;237:346–54.
173. Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, et al. A brain-specific microRNA regulates dendritic spine development. Nature. 2006;439:283–9.
174. Jimenez-Mateos EM, Engel T, Merino-Serrais P, Fernaud-Espinosa I, Rodriguez-Alvarez N, Reynolds J, et al. Antagomirs targeting microRNA-134 increase hippocampal pyramidal neuron spine volume in vivo and protect against pilocarpine-induced status epilepticus. Brain Struct Funct. 2015;220(4):2387–99.
175. Wang XM, Jia RH, Wei D, Cui WY, Jiang W. MiR-134 blockade prevents status epilepticus like-activity and is neuroprotective in cultured hippocampal neurons. Neurosci Lett. 2014;572:20–5.

176. Scholer N, Langer C, Dohner H, Buske C, Kuchenbauer F. Serum microRNAs as a novel class of biomarkers: a comprehensive review of the literature. Exp Hematol. 2010;38:1126–30.
177. De Smaele E, Ferretti E, Gulino A. MicroRNAs as biomarkers for CNS cancer and other disorders. Brain Res. 2010;1338:100–11.
178. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008;18:997–1006.
179. Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, et al. Circulating microRNAs in patients with coronary artery disease. Circ Res. 2010;107:677–84.
180. Corsten MF, Dennert R, Jochems S, Kuznetsova T, Devaux Y, Hofstra L, et al. Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. Circ Cardiovasc Genet. 2010;3:499–506.
181. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9:654–9.
182. Jin XF, Wu N, Wang L, Li J. Circulating microRNAs: a novel class of potential biomarkers for diagnosing and prognosing central nervous system diseases. Cell Mol Neurobiol. 2013;33:601–13.
183. Reid G, Kirschner MB, van Zandwijk N. Circulating microRNAs: association with disease and potential use as biomarkers. Crit Rev Oncol Hematol. 2011;80:193–208.
184. Creemers EE, Tijsen AJ, Pinto YM. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? Circ Res. 2012;110:483–95.
185. Mo MH, Chen L, Fu Y, Wang W, Fu SW. Cell-free circulating miRNA biomarkers in cancer. J Cancer Educ. 2012;3:432–48.
186. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, et al. Treatment of HCV infection by targeting microRNA. N Engl J Med. 2013;368:1685–94.
187. Boudreau RL, Rodriguez-Lebron E, Davidson BL. RNAi medicine for the brain: progresses and challenges. Hum Mol Genet. 2011;20:R21–7.
188. Davidson BL, McCray Jr PB. Current prospects for RNA interference-based therapies. Nat Rev Genet. 2011;12:329–40.

Chapter 5
microRNA and Autism

Ayyappan Anitha and Ismail Thanseem

Abstract Autism is a complex neurodevelopmental disorder characterized by deficiencies in social interaction and communication, and by repetitive and stereotyped behaviors. According to a recent report, the prevalence of this pervasive developmental disorder has risen to 1 in 88. This will have enormous public health implications in the future, and has necessitated the need to discover predictive biomarkers that could index for autism before the onset of symptoms. microRNAs (miRNAs) are small, noncoding RNAs that regulate gene expression at the posttranscriptional level. They have recently emerged as prominent epigenetic regulators of various cellular processes including neurodevelopment. They are abundantly present in the brain, and their dysfunction has been implicated in an array of neuropathological conditions including autism. miRNAs, previously known to be expressed only in cells and tissues, have also been detected in extracellular body fluids such as serum, plasma, saliva, and urine. Altered expression of cellular and circulating miRNAs have been observed in autistic individuals compared to healthy controls. miRNAs are now being considered as potential targets for the development of novel therapeutic strategies for autism.

Keywords Autism • miRNA • Brain • Circulating miRNA

Introduction

Autism spectrum disorders (ASDs) refer to a group of heterogeneous, complex neurodevelopmental disorders characterized by deficiencies in social interaction and communication, and by repetitive and stereotyped behaviors [1]. The abnormalities are usually identified in the early years of childhood, accompanied by impairments in cognitive functioning, learning, attention, and sensory processing. On March 27,

A. Anitha, Ph.D. (✉) • I. Thanseem, Ph.D.
Department of Neurogenetics, Institute for Communicative and Cognitive Neurosciences (ICCONS), Kavalappara, Shoranur, Palakkad, Kerala 679 523, India
e-mail: anitha.a72@gmail.com; thanzim@gmail.com

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_5

2014, the Centers for Disease Control and Prevention (CDC) released new data on the prevalence of autism in the USA. This surveillance study identified 1 in 68 children as having an ASD, which is an alarming proportion. This increased prevalence will have enormous future public health implications, and has necessitated the need to discover predictive noninvasive biomarkers that could index for autism before the onset of symptoms.

Genetic Architecture of Autism Is Complex

Twin and family studies have shown that autism is a highly heritable neuropsychiatric disorder. Much effort has been made to understand the genetic basis of autism and to identify the genetic variants and other chromosomal abnormalities that are responsible for this disorder. However, unlike some of the monogenic disorders which are caused by defects in a single gene, the genetics of autism is very complex, since autism manifests a wide range of symptoms that involves multiple genes. During the last decade, scientists from all over the world have worked together to trace autism candidate genes. Data collected from thousands of autistic families from different populations across the world support a polygenic epistatic model [2].

Linkage analyses and family-based association studies have resulted in the identification of a multitude of autism candidate genes. The recent evolution of SNP microarray and high-throughput sequencing technologies, combined with the availability of large well-characterized patient cohorts has led to a rapidly accumulating pool of genes and loci. Research over the last two decades has shown that autism stems, in large part, from alterations in genes involved in brain development. However, owing to the highly heterogeneous nature of this disorder, the classic genetic studies have not yet been successful in identifying a common susceptibility locus/gene for ASD. In addition to the genetic factors, environmental factors also play a vital role in predisposing individuals to ASD [3].

In recent years, epigenetic mechanisms, which act at the interface of genes and the environment, have been identified as a potential contributor to the pathogenesis of several neurodevelopmental abnormalities including ASD [4]. Epigenetic factors control heritable changes in gene expression without changing the DNA sequence [5].

microRNAs in Neurodevelopment

microRNAs (miRNAs) are prominent epigenetic regulators of various cellular processes including neurodevelopment. They comprise a growing class of evolutionarily conserved, noncoding 15–22 nucleotide RNA molecules that regulate gene expression at the level of translation. miRNAs have been shown to regulate a variety of biological processes, including development, cell proliferation, differentiation, fate specification, growth control, homeostasis, and apoptosis [6, 7].

miRNAs function through a variety of mechanisms that include mRNA degradation and translational repression. The miRNAs bind to the partially complementary regions at the 3′ end of messenger RNAs (mRNAs) stimulating the cleavage or translational repression of corresponding transcripts. Vertebrate genomes are predicted to encode around 2000 unique miRNAs, which could regulate the expression of at least 30 % of genes [8]. As per the most recent reports, a total of 2578 mature miRNAs have been identified in humans [9]. A single miRNA molecule is capable of binding to the recognition site on several mRNAs, subsequently regulating their expression [10]. Thus, miRNA–mRNA interactions can modify the expression of hundreds of target genes, thereby modulating the corresponding cellular and molecular networks. Some key components involved in miRNA processing are shared between lower vertebrates and primates [11–13].

miRNAs are abundantly present in the brain, and have crucial roles in several facets of brain development, neurogenesis, and function [14] as revealed by studies using animal models deficient in miRNA biogenesis [15–18]. Even though ~70 % of the known miRNAs are expressed in the brain, only a few of them are brain-specific and/or brain-enriched [19]. Table 5.1 gives a list of brain-specific and/or brain-enriched miRNAs implicated in crucial neurological functions. Based on high-throughput sequencing data, it has been estimated that >1000 miRNAs are expressed in the human brain [20]. Sempere et al. [21] examined the expression of 119 miRNAs in the mouse and humans organs; 17 miRNAs, known to be involved in neuronal differentiation, maturation, and/or survival, was found to be expressed in the mouse and human brains suggesting their conserved role in neurodevelopment and maintenance. Several of the brain-enriched miRNAs, such as miR-9, miR-34, miR-124, miR-128, miR132, and miR-219, have identical sequences across primates, rodents, *Drosophila*, and *Caenorhabditis elegans* [22–24], indicating that they are highly conserved throughout the evolution. Moreover, there is massive conservation in miRNAs between primates [22, 25]. About 366 out of the 413 miRNAs expressed in human brain have also been detected in rhesus macaque brain [22], making them ideal primate models for the preclinical investigation of miRNAs implicated in neurological dysfunction. In addition, long-term memory processes and the activity of associated synaptic proteins have been found to be modulated by the miRNA RISC pathway in diverse species [26–29]. Thus, miRNAs regulate the development of the nervous system in worms, brain morphogenesis in the fish, and modulate the spatial and temporal expression of crucial genes involved in mammalian brain development [30].

Circulating miRNAs

miRNAs, previously known to be expressed only in cells and tissues, have now been found in extracellular body fluids such as serum, plasma, urine, saliva, amniotic fluid, breast milk, bronchial lavage, cerebrospinal fluid, colostrum, peritoneal fluid, pleural fluid, seminal fluid, and tears in detectable concentrations [31].

Table 5.1 Brain-specific and/or brain-enriched miRNAs involved in neurological functions

miRNA	Function	References
miR-7	Controls neurite outgrowth	[56]
miR-9	Induces neuronal differentiation; affects proliferation and differentiation	[57, 58]
miR-23	Regulates oligodendrocyte development and myelination; regulates cerebral ischemia and neural specification	[59, 60]
miR-124	Promotes neuronal differentiation; regulates spatial learning and social interactions	[61, 62]
miR-125	Regulates synaptic plasticity	[63, 64]
miR-128	Synaptogenesis; regulates apoptosis; inhibits proliferation and self-renewal	[60, 65]
miR-132	Regulator of the brain-to-body resolution of inflammation; contribute to neurodevelopmental and neuromorphological pathologies; neuronal cell development; regulate synaptic plasticity; neuronal maturation; regulates basal and activity-induced neurite outgrowth; regulates recognition memory and synaptic plasticity; regulates circadian clock	[66–73]
miR-134	Controls dendritic spine development, controls synaptic protein synthesis and plasticity, guidance of nerve growth cones, growth-promoting effect on dendritogenesis; inducer of pluripotent stem cell differentiation; stage-specific modulation of cortical development, regulates memory, regulates cholinergic neurotransmission	[27, 74–79]
miR-135	Decreased cell invasion and increased drug sensitivity, regulates Wnt signaling pathway	[21, 80]
miR-137	Neuronal maturation; regulates differentiation of neural stem cells	[81–83]
miR-153	Promote neuronal differentiation	[84]
miR-219	Promotes oligodendrocyte differentiation and myelination, modulates NMDA receptor-mediated neurobehavioral dysfunction, maintenance of lipids and redox homeostasis in mature oligodendrocytes, regulates circadian rhythms of expression.	[85–88]
miR-1202	Associated with the pathophysiology of depression; potential target for antidepressants	[89]

Adapted and modified from [19]

About 90 % of the circulating miRNAs are packaged with proteins (i.e., Argonaute RISC Catalytic Component 2 (Ago2), high-density lipoprotein (HDL), other RNA-binding proteins), while ~10 % are wrapped in small membranous particles (i.e., exosomes, microvesicles, and apoptotic bodies) [32] (Fig. 5.1). Thus they are remarkably stable and resistant to RNase degradation in the biological fluids. However, not much is known about their origin and purpose. It is hypothesized that cell lysis or an increase in the level of exosomes discarded by infected cells contribute to the circulating miRNAs.

Abnormal expression of circulating miRNAs have been reported in several neurological and neurodevelopmental disorders. Recent reports have described the horizontal transfer of biomolecules, including miRNAs, by secreted extracellular

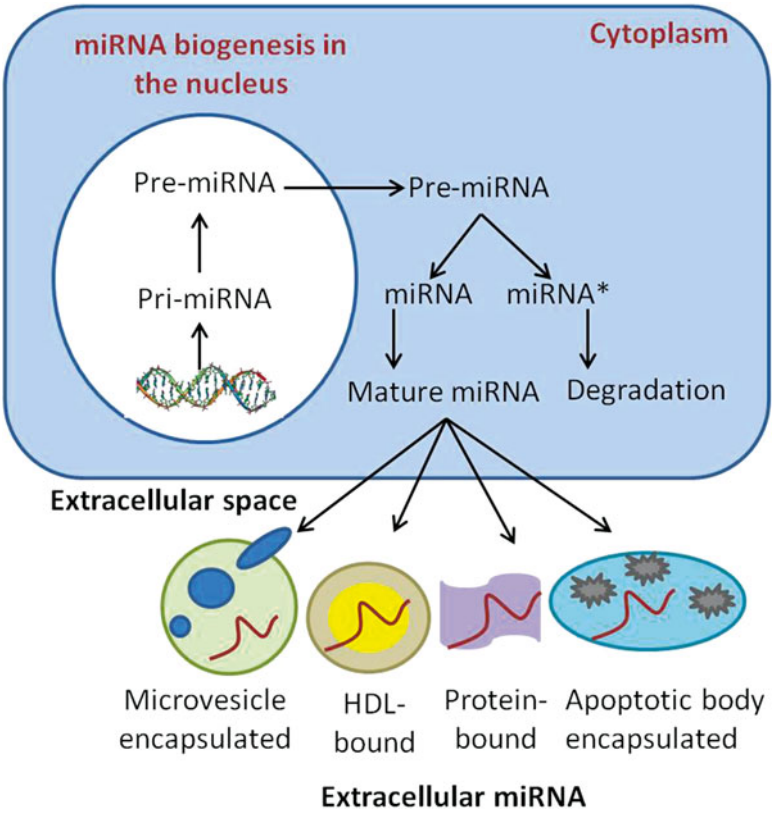

Fig. 5.1 Circulating miRNAs

vesicles, which is increasingly being implicated as a mode of intercellular communication [33]. Thus, miRNAs within the central nervous system (CNS) may be exported across the blood–brain barrier (BBB) by the microvesicles (exosomes) released by neurons and glial cells (Fig. 5.2). Furthermore, the BBB endothelium itself is known to be an important source of circulating miRNAs [34]. The secreted miRNAs could thus be reflective of the pathophysiological processes in the CNS. Moreover, the miRNA expression pattern of brain and peripheral blood lymphocytes has been found to cluster together, indicating that a blood-based expression signature could be a useful biomarker for neurological diseases [35].

miRNAs in Neurological Disorders

Dysregulation of miRNAs has been implicated in several neurological disorders. Studies have shown that the precursor and mature miRNAs, as well as the miRNA processing machinery itself (e.g., Drosha, Dicer), may be dysregulated during the

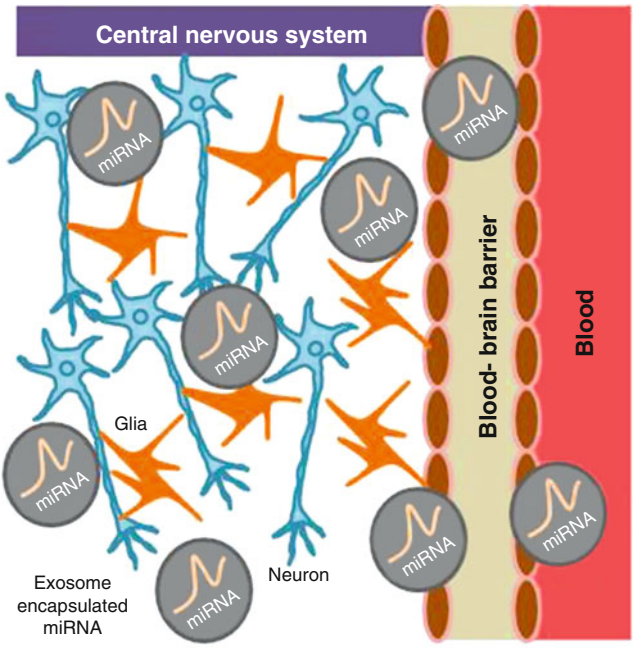

Fig. 5.2 miRNAs may be exported across the blood–brain barrier by microvesicles

progression of a neurological disorder [36–38]. Aberrant expression of miRNAs have been observed in neuropsychiatric disorders such as autism, schizophrenia, bipolar disorder, depression, addiction, and anxiety, as well as in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amylotrophic lateral sclerosis [39]. Certain miRNAs (e.g., miR-132) have been found to be dysregulated in a wide range of psychiatric and neurodegenerative disorders [40–43].

miRNAs in Autism

Aberrant expression of miRNAs have been observed in the postmortem brain [44], lymphoblastoid cell lines [45–47], serum [48], and whole blood [49] of autistic individuals compared to healthy controls (Table 5.2). In a case–control study, Abu-Elneel et al. [44] examined the expression of 466 miRNAs in the postmortem cerebellar cortex of 13 autistic individuals and an equal number of controls. Differential expression of 28 miRNAs was observed in autism brains compared to controls. Talebizadeh et al. [47] compared the miRNA profiles in the lymphoblastoid cell lines (LCL) samples of six individuals with autism and matched control subjects,

Table 5.2 Altered expression of miRNAs in autism

miRNA ID	Biological sample	References
miR-106a, miR-106b, miR-128, miR-129, miR-132, miR-140, miR-146b, miR-148b, miR-15a, miR-15b, miR-181d, miR-193b, miR-21, miR-212, miR-23a, miR-27a, miR-320a, miR-381, miR-431, miR-432, miR-484, miR-539, miR-550, miR-598, miR-652, miR-7, miR-93, miR-95	Cerebellum (postmortem)	[44]
miR-132, miR-146a, miR-146b, miR-23a, miR-23b, miR-320, miR-363, miR-663, miR-92 (a1-a2)	Lymphoblastoid cell lines	[47]
miR-103, miR-106b, miR-107, miR-132, miR-133b, miR-136, miR-139, miR-148b, miR-153-1, miR-16-2, miR-182-AS, miR-185, miR-186, miR-189, miR-190, miR-191, miR-194, miR-195, miR-199a-5p, miR-205, miR-211, miR-219, miR-23a, miR-23b, miR-25, miR-29b, miR-30c, miR-326, miR-342, miR-346, miR-367, miR-376a-AS, miR-451, miR-455, miR-495, miR-518a, miR-519c, miR-520b, miR-524, miR-93	Lymphoblastoid cell lines	[46]
miR-10a, miR-125b, miR-181-b, miR-196a, miR-199a-5p, miR-199b-5p, miR-338-3p, miR-455-3p, miR-486-3p, miR-486-5p, miR-548o, miR-577, miR-650	Lymphoblastoid cell lines	[45]
miR-101-3p, miR-106b-5p, miR-130a-3p, miR-151a-3p, miR-181b-5p, miR-195-5p, miR-19b-3p, miR-320a, miR-328, miR-433, miR-489, miR-572, miR-663a	Serum	[48]
miR-486-3p	Whole blood	[49]

miRNAs with consistent differential expression in two or more studies are shown in bold

and observed an aberrant expression of 9 out of the 470 miRNAs in autism samples. In an independent study using LCL samples, 43 miRNAs were found to be differentially expressed in autistic individuals compared to controls [46]. In yet another study of LCLs, Ghahramani Seno et al. [45] observed an altered expression of 13 miRNAs in the autistic individuals compared to healthy controls. A recent study using serum samples from 55 individuals with autism and 55 age- and sex-matched controls identified 13 miRNAs that were differentially expressed in autistic individuals compared to controls [48]. Popov et al. [49] compared the expression profiles of whole blood miRNAs in 30 autism patients and 25 age- and gender- matched control subjects using a microfluidic array designed to detect 1898 unique mature human miRNA sequences. miR486-3p, a brain-specific miRNA with possible roles in human neuronal differentiation was found to be differentially expressed in autism. Taken together, a few miRNAs (e.g., miR-106-b, miR-132, miR-23a, miR-320a) exhibited a consistent differential expression in autism. Among these, miR-132 has been found to promote the growth and maturation of newborn neurons in the adult hippocampus [50]. Hansen et al. [51] have reported that miR-132 could be pivotal in the regulation of cognition, learning, and memory formation.

Recent studies have implicated copy number variants (CNVs) as a major contributor to the pathogenesis of autism. To date, most of the studies have focused only on the genes present in the CNV loci, with little information on the miRNAs present in these regions. Nearly 11 % of the CNV loci have been found to harbor miRNAs, some of which have already been reported to be associated with autism [52]. The miRNAs present in these deleted and duplicated CNV loci could account for the dosage difference of their target genes.

Enrichment pathway analysis of the target genes of aberrantly expressed miRNAs in autism has revealed several crucial and diverse neurologically relevant pathways. These include the pathways involved in axon guidance, TGF-beta signaling, MAPK signaling, adherens junction, regulation of actin cytoskeleton, oxidative phosphorylation, hedgehog signaling, focal adhesion, MAPK signaling, mTOR signaling, and Wnt signaling [45, 48, 52].

Therapeutic Potential of miRNAs

On the basis of their functional roles in diverse biological pathways, miRNAs appear to be suitable tools for the diagnosis, prognosis, and therapy of several disorders. Accumulating evidence suggests that miRNA-based therapies based on restoring or repressing miRNA expression and/or activity holds great promise. Not all miRNAs have a functional biological role. Cell-based screening strategies making use of suitable platforms such as cell lines, primary cell cultures, differentiated neurons, and induced pluripotent stem cells can facilitate the identification of promising candidates. miRNA-based therapeutics hold a great potential because of their ability to silence multiple genes simultaneously. Due to the complexity of the pathogenic mechanisms underlying the development of neuropsychiatric disorders such as ASDs, miRNAs might be ideally suited for the therapeutic targeting of multiple mRNAs.

miRNA inhibition therapy that downregulates the expression of over-expressed miRNAs using miRNA antagonists, and miRNA replacement therapy that supplements the under-expressed miRNAs using miRNA mimics, are promising approaches in this regard [53]. Despite the great potential, critical hurdles have to be overcome for the success of miRNA-based therapies. These include systemic delivery of targeted miRNAs, poor in vivo stability and inappropriate bio-distribution of delivered miRNA, disruption and saturation of endogenous RNA machinery, and adverse side effects [53]. Viral and nonviral [lipid-based, polyethyleneimine, poly(lactide-co-glycolide), natural polymers, exosomes, nanoparticles] delivery systems have been developed to circumvent these challenges [53]. While the current clinical trials are focused on peripheral organs, therapeutic targeting of the brain remains a future objective.

Future Directions

Pharmacological modulation of disease-associated miRNAs has demonstrated a promising therapeutic potential. Technical developments in miRNA-based therapies for the treatment of cancer, cardiovascular diseases, and HCV infection have come a long way. Clinical phase 1 study of the liposome formulated miR-34 mimic-based drug in liver cancer patients have already commenced [54] and a phase 2 study with antimiR-122 drug has been found to be safe and well tolerated in chronically HCV-infected patients [55]. However, despite compelling evidence for the involvement of miRNAs in neurodevelopment, their contribution to the pathogenesis of ASD has been inadequately assessed to date. A few miRNAs have been identified as consistently dysregulated in autism by several independent studies. If this is an epiphenomenon of the disease, they could be potential candidates in our search for novel biomarkers for this disorder. A systematic characterization of miRNAs targeting high-confidence ASD genes is likely to provide new insights into the mechanisms underlying ASDs. Such an insight may help in designing appropriate miRNA therapeutics for ASDs.

References

1. American Psychiatric Association. Diagnostic and statistical manual of mental disorders DSM-IV-TR. 4th ed. Washington, DC: American Psychiatric Association Publishing Inc; 2000.
2. Persico AM, Napolioni V. Autism genetics. Behav Brain Res. 2013;251:95–112.
3. Meek SE, Lemery-Chalfant K, Jahromi LB, Valiente C. A review of gene environment correlations and their implications for autism: a conceptual model. Psychol Rev. 2013;120:497–521.
4. Miyake K, Hirasawa T, Koide T, Kubota T. Epigenetics in autism and other neurodevelopmental diseases, neurodegenerative diseases. Adv Exp Med Biol. 2012;724:91–8.
5. Delcuve GP, Rastegar M, Davie JR. Epigenetic control. J Cell Physiol. 2009;219:243–50.
6. Martino S, di Girolamo I, Orlacchio A, Datti A, Orlacchio A. MicroRNA implications across neurodevelopment and neuropathology. J Biomed Biotechnol. 2009;2009:654346.
7. Sun E, Shi Y. MicroRNAs: small molecules with big roles in neurodevelopment and diseases. Exp Neurol. 2014;268:46–53.
8. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005;120:15–20.
9. Van Rooij E, Kauppinen S. Development of microRNA therapeutics is coming of age. EMBO Mol Med. 2014;6:851–64.
10. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281–97.
11. Chekulaeva M, Mathys H, Zipprich JT, Attig J, Colic M, et al. miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs. Nat Struct Mol Biol. 2011;18:1218–26.
12. Chekulaeva M, Filipowicz W. Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. Curr Opin Cell Biol. 2009;21:452–60.
13. Park JE, Heo I, Tian Y, Simanshu DK, Chang H, et al. Dicer recognizes the 5′ end of RNA for efficient and accurate processing. Nature. 2011;475:201–5.
14. Kosik KS. The neuronal microRNA system. Nat Rev Neurosci. 2006;7:911–20.

15. Davis TH, Cuellar TL, Koch SM, Barker AJ, Harfe BD, et al. Conditional loss of Dicer disrupts cellular and tissue morphogenesis in the cortex and hippocampus. J Neurosci. 2008;28: 4322–30.
16. De Pietri TD, Pulvers JN, Haffner C, Murchison EP, Hannon GJ, Huttner WB. miRNAs are essential for survival and differentiation of newborn neurons but not for expansion of neural progenitors during early neurogenesis in the mouse embryonic neocortex. Development. 2008;135:3911–21.
17. Giraldez AJ, Cinalli RM, Glasner ME, Enright AJ, Thomson JM, et al. MicroRNAs regulate brain morphogenesis in zebrafish. Science. 2005;308:833–8.
18. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, et al. Argonaute2 is the catalytic engine of mammalian RNAi. Science. 2004;305:1437–41.
19. Adlakha YK, Saini N. Brain microRNAs and insights into biological functions and therapeutic potential of brain enriched miRNA-128. Mol Cancer. 2014;13:33.
20. Berezikov E, Thuemmler F, van Laake LW, Kondova I, Bontrop R, et al. Diversity of microRNAs in human and chimpanzee brain. Nat Genet. 2006;38:1375–7.
21. Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol. 2004;5:R13.
22. Hu HY, Guo S, Xi J, Yan Z, Fu N, et al. MicroRNA expression and regulation in human, chimpanzee, and macaque brains. PLoS Genet. 2011;7, e1002327.
23. Juhila J, Sipilä T, Icay K, Nicorici D, Ellonen P, et al. MicroRNA expression profiling reveals miRNA families regulating specific biological pathways in mouse frontal cortex and hippocampus. PLoS One. 2011;6, e21495.
24. Kapsimali M, Kloosterman WP, de Bruijn E, Rosa F, Plasterk RH, Wilson SW. MicroRNAs show a wide diversity of expression profiles in the developing and mature central nervous system. Genome Biol. 2007;8:R173.
25. Brameier M. Genome-wide comparative analysis of microRNAs in three non-human primates. BMC Res Notes. 2010;3:64.
26. Ashraf SI, McLoon AL, Sclarsic SM, Kunes S. Synaptic protein synthesis associated with memory is regulated by the RISC pathway in Drosophila. Cell. 2006;124:191–205.
27. Gao J, Wang WY, Mao YW, Gräff J, Guan JS, et al. A novel pathway regulates memory and plasticity via SIRT1 and miR-134. Nature. 2010;466:1105–9.
28. Konopka W, Kiryk A, Novak M, Herwerth M, Parkitna JR, et al. MicroRNA loss enhances learning and memory in mice. J Neurosci. 2010;30:14835–42.
29. Rajasethupathy P, Fiumara F, Sheridan R, Betel D, Puthanveettil SV, et al. Characterization of small RNAs in Aplysia reveals a role for miR-124 in constraining synaptic plasticity through CREB. Neuron. 2009;63:803–17.
30. Singh SK. miRNAs: from neurogeneration to neurodegeneration. Pharmacogenomics. 2007;8:971–8.
31. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, et al. The microRNA spectrum in 12 body fluids. Clin Chem. 2010;56:1733–41.
32. Zhu H, Fan G. Extracellular/circulating microRNAs and their potential role in cardiovascular disease. Am J Cardiovasc Dis. 2011;1:138–49.
33. Frühbeis C, Fröhlich D, Krämer-Albers EM. Emerging roles of exosomes in neuron-glia communication. Front Physiol. 2012;3:119.
34. Witwer KW, Sarbanes SL, Liu J, Clements JE. A plasma microRNA signature of acute lentiviral infection: biomarkers of central nervous system disease. AIDS. 2011;25:2057–67.
35. Liang Y, Ridzon D, Wong L, Chen C. Characterization of microRNA expression profiles in normal human tissues. BMC Genomics. 2007;8:166.
36. Beveridge NJ, Gardiner E, Carroll AP, Tooney PA, Cairns MJ. Schizophrenia is associated with an increase in cortical microRNA biogenesis. Mol Psychiatry. 2010;15:1176–89.
37. Fénelon K, Mukai J, Xu B, Hsu PK, Drew LJ, et al. Deficiency of Dgcr8, a gene disrupted by the 22q11.2 microdeletion, results in altered short-term plasticity in the prefrontal cortex. Proc Natl Acad Sci U S A. 2011;108:4447–52.

38. Schofield CM, Hsu R, Barker AJ, Gertz CC, Blelloch R, Ullian EM. Monoallelic deletion of the microRNA biogenesis gene Dgcr8 produces deficits in the development of excitatory synaptic transmission in the prefrontal cortex. Neural Dev. 2011;6:11.
39. Chan AW, Kocerha J. The path to microRNA therapeutics in psychiatric and neurodegenerative disorders. Front Genet. 2012;3:82.
40. Cogswell JP, Ward J, Taylor IA, Waters M, Shi Y, et al. Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. J Alzheimers Dis. 2008;14:27–41.
41. Hollander JA, Im HI, Amelio AL, Kocerha J, Bali P, et al. Striatal microRNA controls cocaine intake through CREB signaling. Nature. 2010;466:197–202.
42. Lee ST, Chu K, Im WS, Yoon HJ, Im JY, et al. Altered microRNA regulation in Huntington's disease models. Exp Neurol. 2011;227:172–9.
43. Perkins DO, Jeffries CD, Jarskog LF, Thomson JM, Woods K, et al. microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. Genome Biol. 2007;8:R27.
44. Abu-Elneel K, Liu T, Gazzaniga FS, Nishimura Y, Wall DP, et al. Heterogeneous dysregulation of microRNAs across the autism spectrum. Neurogenetics. 2008;9:153–61.
45. Ghahramani Seno MM, Hu P, Gwadry FG, Pinto D, Marshall CR, et al. Gene and miRNA expression profiles in autism spectrum disorders. Brain Res. 2011;1380:85–97.
46. Sarachana T, Zhou R, Chen G, Manji HK, Hu VW. Investigation of post-transcriptional gene regulatory networks associated with autism spectrum disorders by microRNA expression profiling of lymphoblastoid cell lines. Genome Med. 2010;2:23.
47. Talebizadeh Z, Butler MG, Theodoro MF. Feasibility and relevance of examining lymphoblastoid cell lines to study role of microRNAs in autism. Autism Res. 2008;1:240–50.
48. Mundalil Vasu M, Anitha A, Thanseem I, Suzuki K, Yamada K, et al. Serum microRNA profiles in children with autism. Mol Autism. 2014;5:40.
49. Popov NT, Madjirova NP, Minkov IN, Vachev VI. Micro RNA hsa-486-3p gene expression profiling in the whole blood of patients with autism. Biotechnol Biotec Equip. 2012;26: 3385–8.
50. Magill ST, Cambronne XA, Luikart BW, Lioy DT, Leighton BH, et al. microRNA-132 regulates dendritic growth and arborization of newborn neurons in the adult hippocampus. Proc Natl Acad Sci U S A. 2010;107:20382–7.
51. Hansen KF, Karelina K, Sakamoto K, Wayman GA, Impey S, et al. miRNA-132: a dynamic regulator of cognitive capacity. Brain Struct Funct. 2013;218:817–31.
52. Vaishnavi V, Manikandan M, Tiwary BK, Munirajan AK. Insights on the functional impact of microRNAs present in autism-associated copy number variants. PLoS One. 2013;8, e56781.
53. Zhang Y, Wang Z, Gameinhart RA. Progress in microRNA delivery. J Control Release. 2013;172:962–74.
54. Bouchie A. First microRNA mimic enters clinic. Nat Biotechnol. 2013;31:577.
55. Janssen HLA, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, et al. Treatment of HCV infection by targeting microRNA. N Engl J Med. 2013;368:1685–94.
56. Chen H, Shalom-Feuerstein R, Riley J, Zhang SD, Tucci P, et al. miR-7 and miR-214 are specifically expressed during neuroblastoma differentiation, cortical development and embryonic stem cells differentiation, and control neurite outgrowth in vitro. Biochem Biophys Res Commun. 2010;394:921–7.
57. Conaco C, Otto S, Han JJ, Mandel G. Reciprocal actions of REST and a microRNA promote neuronal identity. Proc Natl Acad Sci U S A. 2006;103:2422–7.
58. Zhao C, Sun G, Li S, Shi Y. A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. Nat Struct Mol Biol. 2009;16:365–71.
59. Lin ST, Fu YH. miR-23 regulation of lamin B1 is crucial for oligodendrocyte development and myelination. Dis Model Mech. 2009;2:178–88.
60. Smirnova L, Gräfe A, Seiler A, Schumacher S, Nitsch R, Wulczyn FG. Regulation of miRNA expression during neural cell specification. Eur J Neurosci. 2005;21:1469–77.

61. Visvanathan J, Lee S, Lee B, Lee JW, Lee SK. The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. Genes Dev. 2007;21: 744–9.
62. Yang Y, Shu X, Liu D, Shang Y, Wu Y, et al. EPAC null mutation impairs learning and social interactions via aberrant regulation of miR-124 and Zif268 translation. Neuron. 2012;73:774–88.
63. Boissart C, Nissan X, Giraud-Triboult K, Peschanski M, Benchoua A. miR-125 potentiates early neural specification of human embryonic stem cells. Development. 2012;139:1247–57.
64. Edbauer D, Neilson JR, Foster KA, Wang CF, Seeburg DP, et al. Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. Neuron. 2010;65:373–84.
65. Paschou M, Doxakis E. Neurofibromin 1 is a miRNA target in neurons. PLoS One. 2012;7, e46773.
66. Alvarez-Saavedra M, Antoun G, Yanagiya A, Oliva-Hernandez R, Cornejo-Palma D, et al. miRNA-132 orchestrates chromatin remodeling and translational control of the circadian clock. Hum Mol Genet. 2011;20:731–51.
67. Clovis YM, Enard W, Marinaro F, Huttner WB, De Pietri Tonelli D. Convergent repression of Foxp2 3′UTR by miR-9 and miR-132 in embryonic mouse neocortex: implications for radial migration of neurons. Development. 2012;139:3332–42.
68. Miller BH, Zeier Z, Xi L, Lanz TA, Deng S, et al. MicroRNA-132 dysregulation in schizophrenia has implications for both neurodevelopment and adult brain function. Proc Natl Acad Sci U S A. 2012;109:3125–30.
69. Scott HL, Tamagnini F, Narduzzo KE, Howarth JL, Lee YB, et al. MicroRNA-132 regulates recognition memory and synaptic plasticity in the perirhinal cortex. Eur J Neurosci. 2012;36:2941–8.
70. Shaked I, Meerson A, Wolf Y, Avni R, Greenberg D, et al. MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase. Immunity. 2009;31: 965–73.
71. Tognini P, Pizzorusso T. MicroRNA212/132 family: molecular transducer of neuronal function and plasticity. Int J Biochem Cell Biol. 2012;44:6–10.
72. Wanet A, Tacheny A, Arnould T, Renard P. miR-212/132 expression and functions: within and beyond the neuronal compartment. Nucleic Acids Res. 2012;40:4742–53.
73. Wayman GA, Davare M, Ando H, Fortin D, Varlamova O, et al. An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP. Proc Natl Acad Sci U S A. 2008;105:9093–8.
74. Bicker S, Khudayberdiev S, Weiß K, Zocher K, Baumeister S, Schratt G. The DEAH-box helicase DHX36 mediates dendritic localization of the neuronal precursor-microRNA-134. Genes Dev. 2013;27:991–6.
75. Gaughwin P, Ciesla M, Yang H, Lim B, Brundin P. Stage-specific modulation of cortical neuronal development by Mmu-miR-134. Cereb Cortex. 2011;21:1857–69.
76. Han L, Wen Z, Lynn RC, Baudet ML, Holt CE, et al. Regulation of chemotropic guidance of nerve growth cones by microRNA. Mol Brain. 2011;4:40.
77. Meerson A, Cacheaux L, Goosens KA, Sapolsky RM, Soreq H, Kaufer D. Changes in brain MicroRNAs contribute to cholinergic stress reactions. J Mol Neurosci. 2010;40:47–55.
78. Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, et al. A brain-specific microRNA regulates dendritic spine development. Nature. 2006;439:283–9.
79. Tay YM, Tam WL, Ang YS, Gaughwin PM, Yang H, et al. MicroRNA-134 modulates the differentiation of mouse embryonic stem cells, where it causes post-transcriptional attenuation of Nanog and LRH1. Stem Cells. 2008;26:17–29.
80. Sõber S, Laan M, Annilo T. MicroRNAs miR-124 and miR-135a are potential regulators of the mineralocorticoid receptor gene (NR3C2) expression. Biochem Biophys Res Commun. 2010;391:727–32.

81. Smrt RD, Szulwach KE, Pfeiffer RL, Li X, Guo W, et al. MicroRNA miR-137 regulates neuronal maturation by targeting ubiquitin ligase mind bomb-1. Stem Cells. 2010;28:1060–70.
82. Sun G, Ye P, Murai K, Lang MF, Li S, et al. miR-137 forms a regulatory loop with nuclear receptor TLX and LSD1 in neural stem cells. Nat Commun. 2011;2:529.
83. Willemsen MH, Vallès A, Kirkels LA, Mastebroek M, Olde Loohuis N, et al. Chromosome 1p21.3 microdeletions comprising DPYD and MIR137 are associated with intellectual disability. J Med Genet. 2011;48:810–8.
84. Stappert L, Borghese L, Roese-Koerner B, Weinhold S, Koch P, et al. MicroRNA-based promotion of human neuronal differentiation and subtype specification. PLoS One. 2013;8, e59011.
85. Cheng HY, Papp JW, Varlamova O, Dziema H, Russell B, et al. microRNA modulation of circadian-clock period and entrainment. Neuron. 2007;54:813–29.
86. Dugas JC, Cuellar TL, Scholze A, Ason B, Ibrahim A, et al. Dicer1 and miR-219 are required for normal oligodendrocyte differentiation and myelination. Neuron. 2010;65:597–611.
87. Kocerha J, Faghihi MA, Lopez-Toledano MA, Huang J, Ramsey AJ, et al. MicroRNA-219 modulates NMDA receptor-mediated neurobehavioral dysfunction. Proc Natl Acad Sci U S A. 2009;106:3507–12.
88. Zhao X, He X, Han X, Yu Y, Ye F, et al. MicroRNA-mediated control of oligodendrocyte differentiation. Neuron. 2010;65:612–26.
89. Lopez JP, Lim R, Cruceanu C, Crapper L, Fasano C, et al. miR-1202 is a primate-specific and brain-enriched microRNA involved in major depression and antidepressant treatment. Nat Med. 2014;20:764–8.

Chapter 6
microRNAs and Neurodegenerative Diseases

Lifeng Qiu, Eng King Tan, and Li Zeng

Abstract microRNAs (miRNAs) are small, noncoding RNA molecules that through imperfect base-pairing with complementary sequences of target mRNA molecules, typically cleave target mRNA, causing subsequent degradation or translation inhibition. Although an increasing number of studies have identified misregulated miRNAs in the neurodegenerative diseases (NDDs) Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis, which suggests that alterations in the miRNA regulatory pathway could contribute to disease pathogenesis, the molecular mechanisms underlying the pathological implications of misregulated miRNA expression and the regulation of the key genes involved in NDDs remain largely unknown. In this chapter, we provide evidence of the function and regulation of miRNAs and their association with the neurological events in NDDs. This will help improve our understanding of how miRNAs govern the biological functions of key pathogenic genes in these diseases, which potentially regulate several pathways involved in the progression of neurodegeneration. Additionally, given the growing interest in the therapeutic

L. Qiu
Neural Stem Cell Research Lab, Department of Research, National Neuroscience Institute, Singapore 308433, Singapore

E.K. Tan
Department of Neurology, National Neuroscience Institute, SGH Campus, Singapore 169856, Singapore

Department of Research, National Neuroscience Institute, 11 Jalan Tan Tock Seng, Singapore 308433, Singapore

Neuroscience and Behavioral Disorders program, Duke-National University of Singapore, Graduate Medical School, Singapore 169857, Singapore

L. Zeng (✉)
Neural Stem Cell Research Lab, Department of Research, National Neuroscience Institute, Singapore 308433, Singapore

Department of Research, National Neuroscience Institute, 11 Jalan Tan Tock Seng, Singapore 308433, Singapore

Neuroscience and Behavioral Disorders program, Duke-National University of Singapore, Graduate Medical School, Singapore 169857, Singapore
e-mail: Li_ZENG@nni.com.sg

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_6

potential of miRNAs, we discuss current clinical challenges to developing miRNA-based therapeutics for NDDs.

Keywords microRNAs • Neurodegenerative diseases • Alzheimer's disease • NDD • Parkinson's disease • Huntington's disease • Gehrig • ALS • Amyotrophic lateral sclerosis

Introduction

Neurodegenerative diseases (NDDs) are characterized by progressive neuronal degeneration in the central nervous system (CNS). The most prevalent NDDs include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). These diseases are usually incurable but have many similarities, such as protein aggregation, mitochondria dysfunction, and axonal transport defects. Except HD, the majority of cases of the other three NDDs are sporadic, meaning no significant associations with certain genes are detected in these cases. Thus, searching for more general pathological mechanisms is required.

microRNAs (miRNAs) have emerged as an attractive candidate mechanism because they are regulatory molecules that are widely involved in many biological processes. miRNAs are 21- to 24-nucleotide (nt)-long, noncoding RNAs that are transcribed by RNA polymerase II or III as pri-miRNA. Pri-miRNAs are cleaved by Drosha to yield approximately 70- to 100-nt pre-miRNAs [1]. The pre-miRNAs are exported out of the nucleus and are cleaved again by Dicer to generate a 21- to 24-nt mature miRNA. The mature miRNAs form mismatching-permitted complementary binding with the 3′ untranslated region (3′UTR) of the target mRNA through the RNA-induced silencing complex (RISC) [2] (see Chap. 2 of the volume "microRNA: Basic Science" for a detailed discussion of miRNA machinery). Upon binding with their target mRNA, the miRNA's main function is to induce the degradation or translational inhibition of their target mRNAs [2, 3]. Based on computational predictions, one miRNA can downregulate the expression of hundreds of proteins, and this has been experimentally demonstrated [4, 5]. Therefore, the role of miRNA must be considered a crucial aspect in almost every biological process, including the pathogenesis of NDDs.

Brain-enriched miRNAs account for less than one-thirtieth of the total number of identified miRNAs [6, 7], which indicates that the temporal and spatial expression of miRNAs is strictly regulated. miRNAs have been shown to play an important role in neuronal development [8–12]. For example, deletion of Dicer at a stage when ES cells enter the postmitotic state to initiate dopaminergic (DA) neuronal differentiation can completely eliminate DA neuron differentiation [13]. This phenotype can be rescued by introducing small RNA species, including miRNA, into the ES cells, which means that these small RNA species are crucial for DA neuron generation [13]. In addition to neurodevelopment, accumulating evidence has gradually revealed the essential role of miRNA in nervous system morphogenesis, synaptic plasticity, and neurodegeneration [14, 15]. For instance, conditional deletion of Dicer in the adult brain results in obvious neuronal degeneration in the mouse cortex

[15]. All of these findings indicate that miRNA research is becoming increasingly crucial and complicated in neuroscience, including with respect to NDDs.

Here, we summarize the involvement of miRNAs in NDD. We will discuss the dysregulation of miRNAs in each NDD and the roles of specific miRNAs that show promising involvement in these NDDs. Moreover, given that an abnormal increase or decrease in the expression of various miRNAs may contribute to the pathophysiology of NDDs, the use of miRNAs as a therapeutic method to modulate and correct aberrant activity of the nervous system may therefore provide potential clinical treatment for these disorders, which will also be discussed.

miRNA in Neurodegenerative Diseases

miRNAs and AD

Dysregulation of miRNA in AD

Alzheimer's disease (AD) is a neuronal degeneration disease that shows cognitive impairment and dementia clinically [16]. The pathological hallmark of AD is a progressive spread of extracellular Aβ accumulation and intracellular tangles of hyperphosphorylated tau [16]. These pathological proteins in turn cause neuronal loss, starting from the hippocampus and progressing to the entire cortex [16].

miRNA expression profiling has been extensively studied in AD patients, and a number of miRNAs have been shown to be dysregulated in AD patients [17–23]. In human brain, Lukiw found that miR-9 and miR-128 were increased in the hippocampus of AD patients [21]. Cogswell and colleagues identified 8 miRNAs, including miR-9, -26a, -132, and -146b, that were downregulated and 15 miRNAs, including miR-27, -29, -30, -34, and -125b, that were upregulated in the frontal gyrus of AD patients [17]. Hebert and colleagues also detected 13 downregulated miRNAs, including miR-181c, -15a, -9, -101, -29b, -19b, -106b, and -26b, and 3 upregulated miRNAs in the cortex of sporadic AD patients [19]. In human CSF, miR-146b and miR-27a-3p were identified as downregulated [17, 23], and miR-30 family members were upregulated in patients compared to controls [17]. In the cerebral cortex of AD mice (APPswe/PSΔE9 mice), miR-20a, -29a, -125b, -128a, and -106b were downregulated compared to their expression in age-matched controls [18, 22].

miR-9 in AD

Several independent studies have found altered expression of miR-9 in AD brains. However, both upregulation and downregulation have been reported [17, 19, 21, 24–28]. For example, Lukiw and colleagues reported an upregulation of miR-9 in the temporal cortex and hippocampus of AD patients compared to age-matched controls [21, 25]. However, Hebert and colleagues found a decrease in miR-9 in the cortex of sporadic AD patients [19]. In an animal model of AD, miR-9 shows a

significant decrease in the hippocampus of 6-month-old, but not 3-month-old, APPswe/PSΔE9 mice [22].

Because Aβ plaques form in 6-month-old APPswe/PSΔE9 mice, this decreased expression of miR-9 might indicate that miR-9 decreases in response to Aβ accumulation. This hypothesis is consistent with what Schonrock and colleagues found in a cell culture model. They detected miRNA expression changes in response to Aβ treatment in primary neurons [29]. Interestingly, most of the miRNAs that show significant expression changes compared with untreated cells are downregulated in response to Aβ treatment. The proportion of upregulated miRNAs is much smaller. miR-9 is one of the miRNAs that are rapidly downregulated in response to Aβ treatment in primary neurons [29]. miR-9 targets BACE1, which cleaves APP and is a rate-limiting enzyme in Aβ generation [30].

miR-29 in AD

miR-29 was found to be decreased in the brains of sporadic AD patients [19]. Consistently, a decrease in miR-29 has also been observed in the cerebral cortex of APPSwe/PS1ΔE9 mice [22]. miR-29 is an astrocyte-enriched miRNA that is also expressed, to a lesser extent, in mature primary neurons [31, 32]. Its expression dramatically increases with aging [33–35]. The emerging role of miR-29 is to protect cells from apoptosis by targeting and repressing a family of pro-apoptotic proteins, including Bim, Bmf, Hrk, and Puma [31]. Overexpression of miR-29 protects cells from various stimuli-induced cell death. These stimuli include growth factor deprivation, ER stress, and DNA damage [31]. In addition to pro-apoptotic proteins, BACE1 is another confirmed target of miR-29, and the decrease in miR-29 in AD patients is correlated with the increase of BACE1 in these patients [19]. Therefore, decreased expression of miR-29 in AD patients might accelerate Aβ generation by de-repressing BACE1. In summary, miR-29 exhibits a protective role in neuronal survival via both the apoptotic and APP pathways.

miRNA let-7b in AD

miRNA let-7b is increased in the CSF of AD patients compared with controls [36]. This miRNA is involved in the immune response in AD. The increased level of let-7b in the CSF of AD patients might contribute to neurodegeneration by binding to RNA-sensing toll-like receptor (TLR) 7, which is an innate immune receptor [36].

miR-106b in AD

miR-106b, which directly targets APP [33], shows downregulation in both the human AD brain [19, 33] and in the brains of APPSwe/PS1ΔE9 mice [22]. Although it is unclear whether the downregulation of miR-106b is triggered by Aβ

accumulation, decreased expression of miR-106b can possibly enhance the expression of APP, which may accelerate Aβ accumulation.

However, we need to bear in mind that miR-106b might contribute to AD pathology in other ways. One possibility is that miR106b might regulate cell cycle reentry by targeting retinoblastoma protein 1 [37–39] and p21 [37]. Both retinoblastoma protein 1 and p21 are involved in cell cycle regulation. Additionally, miR-106 also targets ITCH, which is an E3 ubiquitin ligase that is involved in the p73 apoptotic signaling pathway [40] and the Wnt signaling pathway [41]. Furthermore, miR-106b can also be involved in autophagy regulation, which is closely related to Aβ accumulation [42], by targeting SQSTM1/p62 [43]. Therefore, miR-106b might participate in AD pathology by regulating APP expression, cell cycle reentry, apoptosis, or even neuronal differentiation.

miRNAs and PD

Dysregulation of miRNA in PD

PD is a neural degenerative disease with symptoms that include tremors,) rigidity, and bradykinesia [44]. The most prominent pathological features are neuronal loss in the substantia nigra [44] and the cellular accumulation of α-synuclein in Lewy bodies [45, 46].

miRNA microarray analyses revealed that miR-34c-5p and miR-637 were significantly downregulated in the amygdala of PD patients compared with controls [47]. Another miRNA, miR-133b, was found to be downregulated in the midbrain of PD patients compared with controls [13]. In peripheral blood mononuclear cells (PBMCs), miR-335, -374a/b, -199, -126, -151-5p, -29b/c, -147, -28-5p, -30b/c, -301a, and -26a were found to be decreased in PD patients compared with controls [48]. In PD *C. elegans* models, miR-64/65 and let-7 were shown to be downregulated compared with controls [49].

miR-133b in PD

In sporadic PD patients, miR-133b is significantly downregulated [13]. miR-133b is a midbrain-enriched miRNA. Overexpression of miR133b moderately promotes the initial stage of DA neuronal differentiation, while inhibiting terminal differentiation, as indicated by the reduced expression of DAT and TH [13]. Dopamine release is also reduced in miR-133b-overexpressing cells. Conversely, inhibition of miR133b in ES cells potentiates DA neuron terminal differentiation and enhances dopamine release [13].

Pituitary homeobox 3 transcription factor (Pitx3) is one of miR-133b's targets [13]. Pitx3 is important not only for DA neuron differentiation but also for the long-term survival of these neurons [50]. Thus, it seems that the decreased expression of

miR-133b in PD patients might participate in PD pathogenesis by affecting DA neuron differentiation. miR-133b and Pitx3 also form a feedback loop in the regulation of DA neuron differentiation. Bioinformatics searches predicted that Pitx3 is one of miR-133b's targets. This was confirmed by a luciferase assay.

However, overexpression of Pitx3 in differentiating ES cells causes an increase in the expression of miR-133b. The regulation of Pitx3 by miR-133b seems direct because the binding between Pitx3 and the promoter of miR-133b was also confirmed by a luciferase assay [13, 51].

miR-107 in PD

Kim and his colleagues reported downregulation of miR-107 in the midbrain of PD patients [13]. One of miR-107's targets is progranulin. Progranulin is a secreted growth factor and also a major genetic cause of frontal temporal dementia [52, 53]. miR-107 is also involved in cell cycle regulation through targeting CDK6, which is important for entry into the G1 phase [54, 55]. As cell cycle reentry commonly leads to cell death in postmeiotic neurons, decreased miR-107 may lead to increased expression of CDK6 and then the promotion of cell cycle reentry, which finally causes cell death. The expression of miR-107 is directly upregulated by p53 [56]. Using a luciferase assay and a ChIP assay, Yamakuchi and colleagues identified a p53 binding site in the 5′UTR of the miR-107's parent gene, pantothenate kinase enzyme 1 (PANK1). Genotoxic stress induces increased expression of miR-107 in a p53-dependent manner [56].

miR-34 in PD

Decreased expression of miR-34 is found in various brain regions of PD patients compared with age-matched controls [22]. The decreased expression of miR-34 in PD patients is an early-stage event, indicating that it is not a secondary response to disease development or drug treatment. It has been shown that the reduction of miR-34 in differentiated SH-SY5Y cells can lead to disruption of the mitochondrial membrane and increased oxidative stress, which in turn leads to decreased cell viability [22].

Bioinformatic studies indicated that Parkin is one of the targets of miR-34; however, this relationship cannot be proven by Western blot analysis. Targets of miR-34 also include SIRT1 [57, 58], Bcl-2 [22], Cdk4, and cyclin D2 [59]. Bcl-2, which is an anti-apoptotic protein, showed an increase in response to miR-34 inhibition and might protect cells from apoptosis. Cdk4 and cyclin D2 are cell cycle regulators that are involved in cell cycle reentry, which triggers cell death in mature neurons [59]. By using a luciferase assay, it was found that p53 can directly bind to the promoter of miR-34a [60]. Conversely, miR-34a can indirectly regulate the expression of p53 by repressing the expression of HDM4, which can

inhibit the expression of p53 by its RING domain [61]. Thus, the double-positive feedback between p53 and miR-34a forms a locked checkpoint to execute the pro-apoptotic process.

miR-205 in PD

In sporadic PD patients, Lrrk2 is considered an important pathogenic factor; Lrrk2 is significantly increased in patients compared with controls [46]. Interestingly, miR-205, which targets the 3′UTR of Lrrk2 and can regulate Lrrk2 expression, was significantly downregulated in the frontal cortex and striatum of sporadic PD patients compared with controls [62].

In contrast, miR-181, -19, and -410, which also contain the 3′UTR binding site of Lrrk2, lacked a significant change in expression in sporadic PD patients. Overexpression of miR-205 in Lrrk2 R1441G mutant primary neurons rescued the shortened neurite phenotype [62]. Interestingly, overexpression of miR-29, which functions in the apoptotic pathway, cannot rescue the phenotype [62], which means that miR-205 works through Lrrk2 in regulating neuronal survival and that the downregulation of miR-205 contributes, at least in part, to the PD pathology induced by elevated Lrrk2 activity [62].

miRNAs and HD

Dysregulated miRNA in HD

HD is a NDD characterized by cognitive and motor defects. It is caused by CAG repeat expansion ,)in the huntingtin (HTT) gene. Pathologically,,) progressive neuronal death is found in the cortex and striatum of HD patients.

Mutant HTT protein, which harbors an expanded polyglutamine tract, has been shown to be associated with Ago2 and P-bodies [63], cytoplasmic sites of RNA metabolism, and RNAi and miRNA activity [64, 65]. Thus, a change in miRNA activity has been proposed to be responsible for the defects in HD. By using RNA sequencing, microarray, and qRT-PCR techniques, Marti et al. found a set of miRNAs that showed dysregulation in the frontal cortex and striatum of HD patients. These miRNAs include miR-100, -151-3p, -16, -219-2-3p, -27b, -451, and -92a, which showed increased expression in the diseased tissue, and miR-128, -139-3p, -222, -382, -433, and -483-3p, which showed decreased expression in the HD patients [66]. Moreover, HTT also interacts with repressor element-1 silencing transcription factor (REST) [67].

Johnson et al. selectively detected changes in the expression of REST-targeting miRNAs, including miR-9, -29a, -29b, -124a, -132, -135b, -139, -203, -204, -212, -330, and -346, in the cortex of R6/2 mice, which are an animal model of HD [68].

They found that four miRNAs, including miR-29a, -124a, -132, and -330, were decreased, while their mRNA targets were correspondingly increased. However, only the change in miR-132 has been further confirmed in the parietal cortical tissue of human patients [68]. Packer et al. also detected the expression change of REST-targeting miRNAs in HD patients, but using Brodmann's areas [69]. They found that the expression of five miRNAs, including miR-9, miR-29b, miR-124a, and miR-132, were significantly changed in HD patients [69].

miR-9 in HD

miR-9-5p and miR-9-3p are both decreased in early HD. As mentioned above, REST, which is a transcription factor that inhibits genes important for neuronal differentiation, is one of the downstream effectors of HTT in HD pathology, and miR-9 is one of the miRNA targets of REST [67, 69]. Aberrant subcellular distribution of REST has been observed in HD [67, 69]. On the other hand, REST and CoREST are also the targets predicted for miR-9-5p and miR-9-3p (TargetScan). Thus, the feedback regulation loop between miR-9 and REST plays an important role in neurogenesis.

Overexpression of miR-9 inhibits the proliferation of neural progenitor cells (NPCs) and promotes neuronal morphogenesis. The pro-neuronal differentiation role of miR-9 is not only mediated by its repression of REST expression but also by its repression of the expression of BAF53a (ACTL6A), which inhibits neurogenesis by regulating chromatin remodeling [70]. Some researchers also claimed that the pro-neurogenesis effect of miR-9 is mediated by the phosphorylation of STAT3 [71]. It is not surprising that miR-9 promotes neurogenesis by regulating various protein targets with regard to the divergent effect of miRNAs on the proteome [5]. The genes that are important for neuronal differentiation are also important for neuronal survival. For example, one of the targets of REST is BDNF, which is essential for neuronal survival. Thus, the decreased expression of miR-9 in HD might contribute to the development of the disease by impairing neurogenesis and neuronal survival.

miR-22 in HD

miR-22 is decreased in HD [69]. Bioinformatic studies indicated that miR-22 targets histone deacetylase 4 (HDAC4), REST co-repressor 1 (Rcor1), and regulator of G-protein signaling 2 (Rgs2) [72]. All three of these genes are important for neurogenesis and neuronal survival and have been implicated in HD pathogenesis. Importantly, the targeting of these three genes by miR-22 has been demonstrated by luciferase assay [72]. Moreover, overexpression of miR-22 in vitro protects neurons against various stresses [72]. miR-22 overexpression has been shown to reduce the activity of caspases, and miR-22 can also directly inhibit the expression of pro-apoptotic proteins, such as mitogen-activated protein kinase 14/p38

(MAPK14/p38) and tumor protein p53-inducible nuclear protein 1 (Tp53inp1). Furthermore, miR-22 can also decrease the accumulation of mutant HTT via an unknown mechanism. Taken together, these results indicate that miR-22 is strongly associated with HD through the neurogenesis and neuronal survival pathway [72].

miR-128 in HD

miR-128 is a neuronally enriched miRNA that plays an important role in neuronal differentiation and survival [73]. It is decreased in the brains of HD mice, monkeys, and humans [66, 69, 74, 75]. In addition to its effect on neurogenesis and neuronal survival, miR-128 is also directly involved in the HTT signaling pathway by targeting HTT, HTT interacting protein 1 (HIP1), SP-1, and GRM5. The targeting of these three genes by miR-128 has been demonstrated by luciferase assay [74]. Thus, miR-128 might be involved in the pathogenesis of HD either by regulating neuronal differentiation and survival or by regulating the HTT signaling pathway.

miR-132 in HD

miR-132 is also downregulated in the cortex of HD patients [75]. miR-132 is another neuronally enriched miRNA. The transcription of miR-132 is directly regulated by CREB and the BDNF pathway, which is known to be crucial in neuronal differentiation and survival [76]. miR-132 can target p250GAP [77], which is important in neurogenesis, and MeCP2 [78], which regulates BDNF expression in the brain. Thus, dysregulation of miR-132 in HD might contribute to disease development by affecting BDNF balance and impairing neurogenesis in the diseased brain.

miRNAs and ALS

Dysregulated miRNA in ALS

ALS is a progressive NDD that affects nerve cells in the brain and spinal cord. Motor neuron,) loss gives rise to malfunctions in muscle,) tissues, causing weakness, atrophy, and ultimately paralysis and death.

Although 90 % of ALS cases are sporadic, a subset of ALS patients is found to carry a TDP-43 mutation [79]. TDP-43 is an RNA binding protein and interacts with Drosha, which is a key miRNA processing enzyme [80, 81]. These findings raise the possibility that TDP-43 may play a role in miRNA processing. In fact, the expression levels of some miRNAs are affected in TDP-43 mutant flies [82]. Furthermore, P-bodies, where RNA metabolism happens, form in response to cellular stress and are central to ALS pathogenesis [83, 84]. Taken together, these findings indicate that miRNAs might be dysregulated in ALS and that the misregulated miRNAs might also be involved in the pathogenesis of ALS.

Using RT-qPCR, Campos-Melo et al. have demonstrated that miR-146, miR-524-5p, and miR-582-3p are dysregulated in the spinal cords of ALS patients. miRNA target analysis has shown that these miRNAs target the 3′UTR of low molecular weight neurofilament (NFL) [85]. Meantime, Koval et al. also identified expression changes of miR-24, miR-142-3p, miR-142-5p, miR-1461, miR-146b, and miR-155 in the spinal cords of both ALS animal models and human patients [86]. Besides the CNS, miRNA dysregulation was also found in the immune system in HD. miRNAs, such as let-7a, let-7b, miR-27a, -146a, -451, -223, -142-5p, and -155, were significantly upregulated in spleen-derived Ly6Chi monocytes both prior to and during the onset of ALS in SOD1 transgenic mice (an ALS mouse model) [87].

miR-206 in ALS

miR-206 is a skeletal muscle-specific miRNA. It is observed to be dramatically increased during the nerve reinnervation process in SOD1 transgenic mice. Consistently, miR-206 knockout worsens the disease and shortens the lives of SOD1 mice [88]. miR-206 was found to induce the secretion of fibroblast growth factor binding protein 1 (FGFBP1) from muscle by inhibiting HDAC4 translation [88]. FGFBP1 can promote presynaptic differentiation at the neuromuscular junction by binding to FGF [89]. Thus, miR-206 is beneficial to heal ALS due to its promotion of synaptogenesis through FGFBP1 signaling.

miR-155 in ALS

miR-155 is increased in both sporadic and familial ALS patients. This increase is harmful because when the increased expression of miR-155 is inhibited in the brains of SOD1G93A rats, the survival of the rats increases [86]. miR-155's function is mostly known in the immune system, where miR-155 was shown to promote tissue inflammation and macrophage inflammatory responses [90]. It has been shown that miR-155 can target SOCS1 mRNA and in turn increase pro-inflammatory cytokine secretion [91]. Furthermore, miR-155 can also downregulate the action of TGF-β, an important cytokine involved in immunosuppression by suppressing two of its targets, SMAD2 [92] and SMAD5 [93].

Therapeutic Potential of miRNAs in Neurodegenerative Diseases

Abnormal increases or decreases in the expression of various miRNAs may contribute to the pathophysiology of NDDs (Table 6.1). The replacement or inhibition of downregulated or upregulated miRNAs may therefore be clinically beneficial in the treatment of these disorders [94–96].

Table 6.1 Summary of miRNAs involved in NDDs

NDDs	miRNAs	Target genes	Biological process involved	References
AD	miR-9	BACE1	APP cleavage	[30]
	miR-29	Bim; Bmf, Hrk; Puma	Neuronal survival	[31]
	let-7b	TLR7(binding)	Immune response	[36]
	miR-106b	APP	APP signaling	[33]
PD	miR-133b	Pitx3	DA neuron differentiation and survival	[13]
	miR-107	Progranulin; CDK6	Neuronal survival	[53–56]
	miR-34	SIRT1; Bcl-2; CDK4	Neuronal survival	[22, 57–59]
	miR-205	Lrrk2	Neuritis outgrowth	[62]
HD	miR-9	REST/CoREST	Neurogenesis	[67, 69]
	miR-22	HDAC4; Rcor1; Rgs2; p38; Tp53inp1	Neurogenesis and neuronal survival	[72]
	miR-128	HTT; HIP1	Neuronal differentiation	[74]
	miR-132	P250GAP; MeCP2	Neurogenesis	[77, 78]
ALS	miR-206	HDAC4	Nerve reinnervation	[88]
	miR-155	SOCS1; TGF-β	Immune response	[91–93]

Most of the progress made in the development of RNA-based therapies has been associated with hereditary NDDs, such as HD [97]. Delivery of a U6 promoter-driven shRNA effector using an adeno-associated virus serotype 1 (AAV1) vector has been shown to target human huntingtin protein, and this treatment could yield improvements in HD-associated neuropathology and behaviors [97]. Although it is difficult to define the appropriate therapeutic targets in sporadic NDDs, recent progress has been made in RNA-based silencing of targets linked to common sporadic forms of PD and AD. For instance, Sapru et al. have successfully targeted the alpha synuclein pathway in the context of PD [98], while in the case of AD, the amyloid [99, 100] and BACE1/β-secretase [101] pathways have both been targeted, with the latter yielding a striking improvement in disease phenotypes.

Development of miRNA-Based Therapeutics

There are two main miRNA-based therapeutic strategies being developed in vivo:

- microRNA mimics
- Anti-microRNAs

miRNA mimics, which are small RNA molecules that resemble miRNA precursors, are used to downregulate the expression of specific target proteins. These proteins can be associated with disease pathogenesis, or with the disease gene itself, and have a gain-of-function pathogenic mutation. The goal of this

strategy is based on the hypothesis that decreasing the level of a specific protein is a protective therapeutic approach.

The second strategy, which has been more widely studied, involves the exploitation of anti-miRNA molecules to create a loss-of-function in the miRNA of interest [102]. In certain disease conditions in which miRNAs are over-expressed, the aim would be to block these miRNAs by introducing a complementary RNA sequence that binds to and inactivates the targeted miRNAs. A major objective in these efforts is to develop oligonucleotides that achieve high in vivo efficacy without significant toxicity.

Development of Anti-microRNA-Based Therapeutics

Antisense Oligonucleotides

Mature miRNAs can be inactivated by administering short complementary synthetic antisense oligonucleotides (ASOs) [103]. Recently, ASOs have been shown to block miRNA precursors and miRNAs without degrading the target RNA [104]. While the complete mechanism behind ASO-mediated inhibition of miRNA activity remains to be determined, anti-miRNA ASOs are likely to be featured as important future therapeutic agents.

Antagomirs

Antagomirs are usually cholesterol-conjugated ASOs that are capable of improving the degradation of specific mRNAs when administered in vivo [105]. To achieve this, Stoffel's group designed "antagomirs," which are RNA snippets conjugated to cholesterol molecules that help the RNA enter into the cells [105]. Intravenous administration of antagomirs results in an apparent decline of corresponding miRNA levels in many organs, such as liver, lung, kidney, heart, and intestine. The silencing of endogenous miRNAs by this method is specific, efficient, and long-lasting. However, antagomirs cannot cross the blood–brain barrier (BBB) but can penetrate brain cells if injected directly into the brain [106].

miRNA Sponge

Another experimental strategy developed in recent years to inhibit miRNA function is to exploit synthetic sponge mRNA, which contains complementary binding sites to a miRNA of interest [107, 108]. When the sponge is expressed from transgenes, it specifically hampers the activity of a family of miRNAs that have a common seed sequence, resulting in the sequestration and blocking of a whole family of related miRNAs [108].

Typical sponge constructs are composed of four to ten binding sites separated by a few nucleotides each. The efficiency of a miRNA sponge depends not only on its affinity for its binding sites but also on need for a high amount of sponge RNAs relative to the amount of the miRNA, which can be achieved by expressing the sponge from a strong promoter, such as the CMV promoter. The sponge method presents several advantages [108]. First, making a sponge to miRNA is easier than knocking it out. Second, many miRNAs have the same seed sequence but are encoded by multiple distant loci; therefore, each locus will have to be knocked out individually and the animals cross-bred to generate a strain with complete knockout. Further, as some miRNA precursors are transcribed in clusters, the deletion of a miRNA within a cluster will be difficult to achieve without affecting others in the same cluster. Because sponges interact with mature miRNAs, their effectiveness is unaffected by the latter two situations. Although sponge technology has advantages in experimental settings, further investigations will be required to develop it into a therapeutically viable strategy.

Locked Nucleic Acid

As the miRNA research field progressed, the first challenge was to develop and improve miRNA detection and functional analysis tools given the small size and sometimes low levels of expression of different miRNAs. An important addition to the miRNA toolbox came from locked nucleic acid (LNA) [109]. LNA-modified oligonucleotides possess high thermal stability when hybridized with their cognate mRNA target molecules and therefore appear to be ideally suited for the targeting of small RNAs, such as miRNAs [109–111]. LNA is a family of conformationally locked nucleotide analogs that are relatively resistant to nuclease activity and may prove to be a suitable platform for the development of miRNA-based therapeutics. For example, it is known that miR-122 regulates cholesterol metabolism and hepatitis C virus (HCV) replication [112]. An LNA-modified ASO blocked miR-122 activity in the liver of African green monkeys without any evidence of toxicity [112], induced long-lasting decreases in plasma cholesterol levels, and decreased HCV levels in the blood of HCV-infected chimpanzees [113].

Development of miRNA Biogenesis-Based Therapeutics

In addition to the development of mimetic and ASOs to modulate miRNA function, recent efforts have also been directed toward developing small-molecule drugs that can influence the biogenesis of miRNAs or directly influence their function [114]. The small-molecule anti-inflammatory drug enoxacin (Penetrex™) promotes the biogenesis of endogenous miRNAs [115–117]. Conversely, compounds that disrupt miRNA biogenesis have been identified [118]. It will be important to determine if similar screening approaches can be used to identify chemical probes and potentially useful drugs to selectively modulate the activity of miRNAs involved in NDDs.

Challenge of Delivering Therapeutic miRNA Targets in NDDs

The use of miRNAs as potential therapeutic targets remains controversial with regard to methods of delivery and target specificity. When considering a treatment for NDDs that is mediated by miRNA delivery, we have to assess its ability to pass through the BBB as well as its neurotoxicity and off-target effects. To overcome the problem of the BBB, several siRNA delivery systems are being developed for in vivo purposes. These include vector-based, chemically modified, and "packaged" RNA oligonucleotides [119].

Crossing the BBB to Deliver Therapeutics miRNA Targets

Exosome

A major drawback in delivering ASOs is the relative difficulty of crossing the BBB. Intriguingly, systemically injected exosomes, which are small transport vesicles secreted by many classes of mammalian cells, can be used to selectivity deliver small RNAs to the brain in mice [120]. Exosomes are cell-derived vesicles that enable cell-to-cell communication by transferring RNA molecules and proteins [120]. They have been shown to preserve mRNAs and miRNAs in the presence of RNase and to subsequently deliver them to recipient cells [121]. In particular, Alvarez-Erviti et al. isolated brain-targeting exosomes from dendritic cells bioengineered to express an exosomal membrane protein (lamp 2b) fused to a ligand of the acetylcholine receptor. Exosomes were then loaded with siRNAs targeting BACE1 mRNA by electroporation and injected intravenously, resulting in a significant knockdown of BACE1 expression [120]. Using this approach as a future therapeutic vehicle to deliver siRNA/miRNA is worthy for several reasons, including the specific targeting of exosomes to the brain following systemic delivery, the ability to load the desired siRNA/miRNA into the exosomes, and the ability to escape an immune response.

Non-viral

To avoid the action of nucleases, chemical modification or non-viral carriers can be used [122]. Conjugation with non-viral carriers might induce marked toxicity because RNA molecules will also enter the non-targeted cells due to an interaction between the negatively charged cellular membrane and cationic carriers [123]. After RNA molecules have passed these physiological barriers, they have to enter the target cells to elicit their effects. This means that they have to cross the cell membrane, escape endosomes, and localize to the nucleus. Therefore, nuclear-localization signals and cell-penetrating and endosomal-release signal peptides can influence the duration of action of injected RNA molecules [124].

Viral

Adenoviral, lentiviral, and adeno-associated virus-based local delivery has also been performed in animal models of AD and HD [97], demonstrating significant improvement. Nevertheless, the strategy to locally deliver a drug to the brain is still far from normal practice because of the complexities associated with direct injection into the human brain.

Toxicity and Off-Target Effects of Delivering Therapeutic miRNA Targets

There are considerable challenges to the clinical application of exogenous RNAi effector sequences. A number of toxicities are associated with siRNAs and exogenous RNAi precursors. High levels of expressed shRNAs in the liver are known to cause fatalities in mice due, in part, to saturation of the endogenous RNAi machinery [125], and McBride and colleagues have observed toxicity arising from shRNA-based vectors in the brain [126, 127]. Expressed shRNAs likely abrogate the function of natural miRNAs [125, 128, 129]. Therefore, careful consideration should be given to the dosage used when applying ectopically introduced RNAi effectors. Another area of concern is the possibility of off-target inhibition of unintended mRNAs through interactions between the 6- to 7-nt seed region of the exogenous siRNA guide sequences and the target mRNAs. Many off-target effects of this nature have been observed when introducing exogenous siRNAs [130–132]. Off-target effects can in some instances be mitigated by introducing specific base modifications within the siRNA duplex [133]. However, to determine the full extent of any off-target inhibition, prior screening using candidate RNA and protein expression array analyses may be required [5].

Conclusion

In summary, the current evidence clearly points to a significant role for miRNAs in NDDs. miRNAs are of particular interest in understanding these complexes of disorders because they can potentially regulate several pathways involved in the onset and progression of these diseases. The study of miRNA appears particularly promising for understanding the very prevalent but poorly understood sporadic forms of NDDs, such as AD and PD. The challenge now is to understand the role of specific miRNAs in biological models and to translate this knowledge to clinical studies. Moving forward, the development of tools for the delivery, stable expression and controlled modulation of miRNA levels and their action in vivo will be a valuable asset in enhancing the therapeutic value of miRNAs in NDDs.

References

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2): 281–97.
2. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2): 215–33.
3. Ambros V. The functions of animal microRNAs. Nature. 2004;431(7006):350–5.
4. Lim LP, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature. 2005;433(7027):769–73.
5. Selbach M, et al. Widespread changes in protein synthesis induced by microRNAs. Nature. 2008;455(7209):58–63.
6. Bak M, et al. MicroRNA expression in the adult mouse central nervous system. RNA. 2008;14(3):432–44.
7. Landgraf P, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell. 2007;129(7):1401–14.
8. Davis TH, et al. Conditional loss of Dicer disrupts cellular and tissue morphogenesis in the cortex and hippocampus. J Neurosci. 2008;28(17):4322–30.
9. Giraldez AJ, et al. MicroRNAs regulate brain morphogenesis in zebrafish. Science. 2005;308(5723):833–8.
10. Kawase-Koga Y, et al. RNAase-III enzyme Dicer maintains signaling pathways for differentiation and survival in mouse cortical neural stem cells. J Cell Sci. 2010;123(Pt 4):586–94.
11. Kosik KS. The neuronal microRNA system. Nat Rev Neurosci. 2006;7(12):911–20.
12. McLoughlin HS, et al. Dicer is required for proliferation, viability, migration and differentiation in corticoneurogenesis. Neuroscience. 2012;223:285–95.
13. Kim J, et al. A MicroRNA feedback circuit in midbrain dopamine neurons. Science. 2007;317(5842):1220–4.
14. Haramati S, et al. miRNA malfunction causes spinal motor neuron disease. Proc Natl Acad Sci U S A. 2010;107(29):13111–6.
15. Hebert SS, et al. Genetic ablation of Dicer in adult forebrain neurons results in abnormal tau hyperphosphorylation and neurodegeneration. Hum Mol Genet. 2010;19(20):3959–69.
16. Karch CM, Goate AM. Alzheimer's disease risk genes and mechanisms of disease pathogenesis. Biol Psychiatry. 2015;77(1):43–51.
17. Cogswell JP, et al. Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. J Alzheimers Dis. 2008;14(1):27–41.
18. Delay C, Hebert SS. MicroRNAs and Alzheimer's disease mouse models: current insights and future research avenues. Int J Alzheimers Dis. 2011;2011:894938.
19. Hebert SS, et al. Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. Proc Natl Acad Sci U S A. 2008;105(17):6415–20.
20. Leidinger P, et al. A blood based 12-miRNA signature of Alzheimer disease patients. Genome Biol. 2013;14(7):R78.
21. Lukiw WJ. Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus. Neuroreport. 2007;18(3):297–300.
22. Wang X, et al. miR-34a, a microRNA up-regulated in a double transgenic mouse model of Alzheimer's disease, inhibits bcl2 translation. Brain Res Bull. 2009;80(4-5):268–73.
23. Sala Frigerio C, et al. Reduced expression of hsa-miR-27a-3p in CSF of patients with Alzheimer disease. Neurology. 2013;81(24):2103–6.
24. Nunez-Iglesias J, et al. Joint genome-wide profiling of miRNA and mRNA expression in Alzheimer's disease cortex reveals altered miRNA regulation. PLoS One. 2010;5(2), e8898.
25. Sethi P, Lukiw WJ. Micro-RNA abundance and stability in human brain: specific alterations in Alzheimer's disease temporal lobe neocortex. Neurosci Lett. 2009;459(2):100–4.

26. Wang WX, et al. Patterns of microRNA expression in normal and early Alzheimer's disease human temporal cortex: white matter versus gray matter. Acta Neuropathol. 2011;121(2): 193–205.
27. Wang WX, et al. The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1. J Neurosci. 2008;28(5):1213–23.
28. Wang Y, et al. Transforming growth factor-beta regulates the sphere-initiating stem cell-like feature in breast cancer through miRNA-181 and ATM. Oncogene. 2011;30(12):1470–80.
29. Schonrock N, et al. Neuronal microRNA deregulation in response to Alzheimer's disease amyloid-beta. PLoS One. 2010;5(6), e11070.
30. Yan R, Vassar R. Targeting the beta secretase BACE1 for Alzheimer's disease therapy. Lancet Neurol. 2014;13(3):319–29.
31. Kole AJ, et al. miR-29b is activated during neuronal maturation and targets BH3-only genes to restrict apoptosis. Genes Dev. 2011;25(2):125–30.
32. Smirnova L, et al. Regulation of miRNA expression during neural cell specification. Eur J Neurosci. 2005;21(6):1469–77.
33. Hebert SS, et al. MicroRNA regulation of Alzheimer's Amyloid precursor protein expression. Neurobiol Dis. 2009;33(3):422–8.
34. Ling KH, et al. Deep sequencing analysis of the developing mouse brain reveals a novel microRNA. BMC Genomics. 2011;12:176.
35. Somel M, et al. MicroRNA, mRNA, and protein expression link development and aging in human and macaque brain. Genome Res. 2010;20(9):1207–18.
36. Lehmann SM, et al. An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. Nat Neurosci. 2012;15(6):827–35.
37. Ivanovska I, et al. MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Mol Cell Biol. 2008;28(7):2167–74.
38. Landais S, et al. Oncogenic potential of the miR-106-363 cluster and its implication in human T-cell leukemia. Cancer Res. 2007;67(12):5699–707.
39. Trompeter HI, et al. MicroRNAs MiR-17, MiR-20a, and MiR-106b act in concert to modulate E2F activity on cell cycle arrest during neuronal lineage differentiation of USSC. PLoS One. 2011;6(1), e16138.
40. Sampath D, et al. Specific activation of microRNA106b enables the p73 apoptotic response in chronic lymphocytic leukemia by targeting the ubiquitin ligase Itch for degradation. Blood. 2009;113(16):3744–53.
41. Shen G, et al. miR-106b downregulates adenomatous polyposis coli and promotes cell proliferation in human hepatocellular carcinoma. Carcinogenesis. 2013;34(1):211–9.
42. Nilsson P, et al. Abeta secretion and plaque formation depend on autophagy. Cell Rep. 2013;5(1):61–9.
43. Meenhuis A, et al. MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice. Blood. 2011;118(4):916–25.
44. Savitt JM, Dawson VL, Dawson TM. Diagnosis and treatment of Parkinson disease: molecules to medicine. J Clin Invest. 2006;116(7):1744–54.
45. Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neuron. 2003; 39(6):889–909.
46. Dawson TM, Dawson VL. Molecular pathways of neurodegeneration in Parkinson's disease. Science. 2003;302(5646):819–22.
47. Minones-Moyano E, et al. MicroRNA profiling of Parkinson's disease brains identifies early downregulation of miR-34b/c which modulate mitochondrial function. Hum Mol Genet. 2011;20(15):3067–78.
48. Martins M, et al. Convergence of miRNA expression profiling, alpha-synuclein interaction and GWAS in Parkinson's disease. PLoS One. 2011;6(10), e25443.
49. Asikainen S, et al. Global microRNA expression profiling of Caenorhabditis elegans Parkinson's disease models. J Mol Neurosci. 2010;41(1):210–8.

50. Li J, Dani JA, Le W. The role of transcription factor Pitx3 in dopamine neuron development and Parkinson's disease. Curr Top Med Chem. 2009;9(10):855–9.
51. Mouradian MM. MicroRNAs in Parkinson's disease. Neurobiol Dis. 2012;46(2):279–84.
52. van Swieten JC, Heutink P. Mutations in progranulin (GRN) within the spectrum of clinical and pathological phenotypes of frontotemporal dementia. Lancet Neurol. 2008;7(10): 965–74.
53. Wang WX, et al. miR-107 regulates granulin/progranulin with implications for traumatic brain injury and neurodegenerative disease. Am J Pathol. 2010;177(1):334–45.
54. Feng L, et al. miR-107 targets cyclin-dependent kinase 6 expression, induces cell cycle G1 arrest and inhibits invasion in gastric cancer cells. Med Oncol. 2012;29(2):856–63.
55. Takahashi Y, et al. MiR-107 and MiR-185 can induce cell cycle arrest in human non small cell lung cancer cell lines. PLoS One. 2009;4(8), e6677.
56. Yamakuchi M, et al. P53-induced microRNA-107 inhibits HIF-1 and tumor angiogenesis. Proc Natl Acad Sci U S A. 2010;107(14):6334–9.
57. Yamakuchi M, Ferlito M, Lowenstein CJ. miR-34a repression of SIRT1 regulates apoptosis. Proc Natl Acad Sci U S A. 2008;105(36):13421–6.
58. Filatova EV, et al. MicroRNAs: possible role in pathogenesis of Parkinson's disease. Biochemistry (Mosc). 2012;77(8):813–9.
59. Antonini D, et al. Transcriptional repression of miR-34 family contributes to p63-mediated cell cycle progression in epidermal cells. J Invest Dermatol. 2010;130(5):1249–57.
60. Chang TC, et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell. 2007;26(5):745–52.
61. Okada N, et al. A positive feedback between p53 and miR-34 miRNAs mediates tumor suppression. Genes Dev. 2014;28(5):438–50.
62. Cho HJ, et al. MicroRNA-205 regulates the expression of Parkinson's disease-related leucine-rich repeat kinase 2 protein. Hum Mol Genet. 2013;22(3):608–20.
63. Savas JN, et al. Huntington's disease protein contributes to RNA-mediated gene silencing through association with Argonaute and P bodies. Proc Natl Acad Sci U S A. 2008;105(31):10820–5.
64. Jagannath A, Wood MJ. Localization of double-stranded small interfering RNA to cytoplasmic processing bodies is Ago2 dependent and results in up-regulation of GW182 and Argonaute-2. Mol Biol Cell. 2009;20(1):521–9.
65. Jakymiw A, et al. Disruption of GW bodies impairs mammalian RNA interference. Nat Cell Biol. 2005;7(12):1267–74.
66. Marti E, et al. A myriad of miRNA variants in control and Huntington's disease brain regions detected by massively parallel sequencing. Nucleic Acids Res. 2010;38(20):7219–35.
67. Zuccato C, et al. Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. Nat Genet. 2003;35(1):76–83.
68. Johnson R, et al. A microRNA-based gene dysregulation pathway in Huntington's disease. Neurobiol Dis. 2008;29(3):438–45.
69. Packer AN, et al. The bifunctional microRNA miR-9/miR-9* regulates REST and CoREST and is downregulated in Huntington's disease. J Neurosci. 2008;28(53):14341–6.
70. Yoo AS, et al. MicroRNA-mediated switching of chromatin-remodelling complexes in neural development. Nature. 2009;460(7255):642–6.
71. Krichevsky AM, et al. Specific microRNAs modulate embryonic stem cell-derived neurogenesis. Stem Cells. 2006;24(4):857–64.
72. Jovicic A, et al. MicroRNA-22 (miR-22) overexpression is neuroprotective via general anti-apoptotic effects and may also target specific Huntington's disease-related mechanisms. PLoS One. 2013;8(1), e54222.
73. Guidi M, et al. Overexpression of miR-128 specifically inhibits the truncated isoform of NTRK3 and upregulates BCL2 in SH-SY5Y neuroblastoma cells. BMC Mol Biol. 2010;11:95.
74. Kocerha J, et al. microRNA-128a dysregulation in transgenic Huntington's disease monkeys. Mol Brain. 2014;7:46.

75. Lee ST, et al. Altered microRNA regulation in Huntington's disease models. Exp Neurol. 2011;227(1):172–9.
76. Remenyi J, et al. Regulation of the miR-212/132 locus by MSK1 and CREB in response to neurotrophins. Biochem J. 2010;428(2):281–91.
77. Vo N, et al. A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. Proc Natl Acad Sci U S A. 2005;102(45):16426–31.
78. Klein ME, et al. Homeostatic regulation of MeCP2 expression by a CREB-induced microRNA. Nat Neurosci. 2007;10(12):1513–4.
79. Sreedharan J, et al. TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. Science. 2008;319(5870):1668–72.
80. Han J, et al. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev. 2004;18(24):3016–27.
81. Kawahara Y, Mieda-Sato A. TDP-43 promotes microRNA biogenesis as a component of the Drosha and Dicer complexes. Proc Natl Acad Sci U S A. 2012;109(9):3347–52.
82. Buratti E, et al. Nuclear factor TDP-43 can affect selected microRNA levels. FEBS J. 2010;277(10):2268–81.
83. Pare JM, Lopez-Orozco J, Hobman TC. MicroRNA-binding is required for recruitment of human Argonaute 2 to stress granules and P-bodies. Biochem Biophys Res Commun. 2011;414(1):259–64.
84. Ramaswami M, Taylor JP, Parker R. Altered ribostasis: RNA-protein granules in degenerative disorders. Cell. 2013;154(4):727–36.
85. Campos-Melo D, et al. Altered microRNA expression profile in Amyotrophic Lateral Sclerosis: a role in the regulation of NFL mRNA levels. Mol Brain. 2013;6:26.
86. Koval ED, et al. Method for widespread microRNA-155 inhibition prolongs survival in ALS-model mice. Hum Mol Genet. 2013;22(20):4127–35.
87. Butovsky O, et al. Modulating inflammatory monocytes with a unique microRNA gene signature ameliorates murine ALS. J Clin Invest. 2012;122(9):3063–87.
88. Williams AH, et al. MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. Science. 2009;326(5959):1549–54.
89. Fox MA, et al. Distinct target-derived signals organize formation, maturation, and maintenance of motor nerve terminals. Cell. 2007;129(1):179–93.
90. O'Connell RM, Rao DS, Baltimore D. microRNA regulation of inflammatory responses. Annu Rev Immunol. 2012;30:295–312.
91. Wang P, et al. Inducible microRNA-155 feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. J Immunol. 2010;185(10):6226–33.
92. Louafi F, Martinez-Nunez RT, Sanchez-Elsner T. MicroRNA-155 targets SMAD2 and modulates the response of macrophages to transforming growth factor-{beta}. J Biol Chem. 2010;285(53):41328–36.
93. Rai D, et al. Targeting of SMAD5 links microRNA-155 to the TGF-beta pathway and lymphomagenesis. Proc Natl Acad Sci U S A. 2010;107(7):3111–6.
94. Qiu L, et al. Deciphering the function and regulation of microRNAs in Alzheimer's disease and Parkinson's disease. ACS Chem Neurosci. 2014;5(10):884–94.
95. Liu XQ, et al. Targeted delivery of antisense inhibitor of miRNA for antiangiogenesis therapy using cRGD-functionalized nanoparticles. Mol Pharm. 2011;8(1):250–9.
96. Wiggins JF, et al. Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34. Cancer Res. 2010;70(14):5923–30.
97. Harper SQ, et al. RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. Proc Natl Acad Sci U S A. 2005;102(16):5820–5.
98. Sapru MK, et al. Silencing of human alpha-synuclein in vitro and in rat brain using lentiviral-mediated RNAi. Exp Neurol. 2006;198(2):382–90.
99. Miller VM, et al. Targeting Alzheimer's disease genes with RNA interference: an efficient strategy for silencing mutant alleles. Nucleic Acids Res. 2004;32(2):661–8.

100. Xie Z, et al. RNA interference silencing of the adaptor molecules ShcC and Fe65 differentially affect amyloid precursor protein processing and Abeta generation. J Biol Chem. 2007; 282(7):4318–25.
101. Singer O, et al. Targeting BACE1 with siRNAs ameliorates Alzheimer disease neuropathology in a transgenic model. Nat Neurosci. 2005;8(10):1343–9.
102. Meister G, et al. Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. RNA. 2004;10(3):544–50.
103. Hutvagner G, et al. Sequence-specific inhibition of small RNA function. PLoS Biol. 2004;2(4), E98.
104. Michlewski G, et al. Posttranscriptional regulation of miRNAs harboring conserved terminal loops. Mol Cell. 2008;32(3):383–93.
105. Krutzfeldt J, et al. Silencing of microRNAs in vivo with 'antagomirs'. Nature. 2005;438(7068):685–9.
106. Kuhn DE, et al. Chromosome 21-derived microRNAs provide an etiological basis for aberrant protein expression in human Down syndrome brains. J Biol Chem. 2010;285(2): 1529–43.
107. Care A, et al. MicroRNA-133 controls cardiac hypertrophy. Nat Med. 2007;13(5):613–8.
108. Ebert MS, Neilson JR, Sharp PA. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. Nat Methods. 2007;4(9):721–6.
109. Kumar R, et al. The first analogues of LNA (locked nucleic acids): phosphorothioate-LNA and 2′-thio-LNA. Bioorg Med Chem Lett. 1998;8(16):2219–22.
110. Braasch DA, Corey DR. Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. Chem Biol. 2001;8(1):1–7.
111. Kurreck J, et al. Design of antisense oligonucleotides stabilized by locked nucleic acids. Nucleic Acids Res. 2002;30(9):1911–8.
112. Elmen J, et al. LNA-mediated microRNA silencing in non-human primates. Nature. 2008;452(7189):896–9.
113. Lanford RE, et al. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science. 2010;327(5962):198–201.
114. Li Y, He C, Jin P. Emergence of chemical biology approaches to the RNAi/miRNA pathway. Chem Biol. 2010;17(6):584–9.
115. Melo S, et al. Small molecule enoxacin is a cancer-specific growth inhibitor that acts by enhancing TAR RNA-binding protein 2-mediated microRNA processing. Proc Natl Acad Sci U S A. 2011;108(11):4394–9.
116. Shan G, et al. A small molecule enhances RNA interference and promotes microRNA processing. Nat Biotechnol. 2008;26(8):933–40.
117. Zhang Q, Zhang C, Xi Z. Enhancement of RNAi by a small molecule antibiotic enoxacin. Cell Res. 2008;18(10):1077–9.
118. Watashi K, et al. Identification of small molecules that suppress microRNA function and reverse tumorigenesis. J Biol Chem. 2010;285(32):24707–16.
119. Kim DH, Rossi JJ. Strategies for silencing human disease using RNA interference. Nat Rev Genet. 2007;8(3):173–84.
120. Alvarez-Erviti L, et al. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol. 2011;29(4):341–5.
121. Valadi H, et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6):654–9.
122. Borchard G. Chitosans for gene delivery. Adv Drug Deliv Rev. 2001;52(2):145–50.
123. Uyechi LS, et al. Mechanism of lipoplex gene delivery in mouse lung: binding and internalization of fluorescent lipid and DNA components. Gene Ther. 2001;8(11):828–36.
124. Jere D, et al. Bioreducible polymers for efficient gene and siRNA delivery. Biomed Mater. 2009;4(2):025020.
125. Grimm D, et al. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature. 2006;441(7092):537–41.

126. Boudreau RL, Martins I, Davidson BL. Artificial microRNAs as siRNA shuttles: improved safety as compared to shRNAs in vitro and in vivo. Mol Ther. 2009;17(1):169–75.
127. McBride JL, et al. Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. Proc Natl Acad Sci U S A. 2008;105(15):5868–73.
128. Keck K, et al. Rational design leads to more potent RNA interference against hepatitis B virus: factors effecting silencing efficiency. Mol Ther. 2009;17(3):538–47.
129. An DS, et al. Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. Mol Ther. 2006;14(4):494–504.
130. Jackson AL, et al. Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. RNA. 2006;12(7):1179–87.
131. Scacheri PC, et al. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. Proc Natl Acad Sci U S A. 2004;101(7):1892–7.
132. Burchard J, et al. MicroRNA-like off-target transcript regulation by siRNAs is species specific. RNA. 2009;15(2):308–15.
133. Jackson AL, et al. Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. RNA. 2006;12(7):1197–205.

Chapter 7
microRNAs and Fragile X Syndrome

Shi-Lung Lin

Abstract Fragile X syndrome (FXS) is one of the major causes for autism and mental retardation in humans. The etiology of FXS is linked to the expansion of the CGG trinucleotide repeats, r(CGG), suppressing the *fragile X mental retardation 1* (*FMR1*) gene on the X chromosome, resulting in a loss of fragile X mental retardation protein (FMRP) expression, which is required for regulating normal neuronal connectivity and plasticity. Recent studies have further identified that microRNAs are involved in the mechanisms underlying FXS pathogenesis at three different developmental stages. During early embryogenesis before the blastocyst stage, an embryonic stem cell (ESC)-specific microRNA, miR-302, interferes with *FMR1* mRNA translation to maintain the stem cell status and inhibit neural development. After blastocyst, the downregulation of miR-302 releases FMRP synthesis and subsequently leads to neuronal development; yet, in FXS, certain r(CGG)-derived microRNAs, such as miR-fmr1s, are expressed and accumulated and then induce DNA hypermethylation on the *FMR1* gene promoter regions, resulting in transcriptional inactivation of the *FMR1* gene and the loss of FMRP. In normal neuronal development, FMRP is an RNA-binding protein responsible for interacting with miR-125 and miR-132 to regulate the signaling of Group 1 metabotropic glutamate receptor (mGluR1) and *N*-methyl-D-aspartate receptor (NMDAR), respectively, and consequently affecting synaptic plasticity. As a result, the loss of FMRP impairs these signaling controls and eventually causes FXS-associated disorders, such as autism and mental retardation. Based on these current findings, this chapter will summarize the etiological causes of FXS and further provides significant insights into the molecular mechanisms underlying microRNA-mediated FXS pathogenesis and the related therapy development.

Keywords microRNA • Fragile X syndrome • FMR1 • FMRP • RNAi • RITS

S.-L. Lin (✉)
Division of Regenerative Medicine, WJWU & LYNN Institute for Stem Cell Research, 12145 Mora Drive, STE6, Santa Fe Springs, CA 90670, USA
e-mail: shilungl@mirps.org

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_7

Introduction

A large portion of the genome is noncoding DNA, which often contains microsatellite-like short nucleotide repeats with unknown function. Recent studies have shown that the transcripts of certain trinucleotide repeats can fold into hairpin-like RNAs, which are then further processed by RNaseIII-associated *Dicer*s into microRNAs (miRNAs) or miRNA-like gene silencing molecules that are sized about 18–27 nucleotides and capable of interfering with specific target genes through either posttranscriptional mRNA degradation or translational suppression, or both [1–5].

These miRNAs play crucial roles in several triplet repeat expansion diseases (TREDs), including fragile X syndrome (FXS), Huntington's disease (HD), myotonic dystrophy (DM), and a number of spinocerebellar ataxias (SCAs). Yet, most of the pathogenic mechanisms underlying these diseases remain largely unclear. To this, we will use FXS as a model to demonstrate how miRNAs are involved in the pathogenesis of FXS-associated disorders, such as autism and mental retardation.

FXS is one of the most common forms of inherited mental retardation, taking up approximately 30 % of total human mental retardation disorders [6]. This disease is originated from the transcriptional inactivation of *fragile X mental retardation 1* (*FMR1*) gene and thus losing its encoded protein, FMRP. FMRP is associated with polyribosome assembly to form ribonucleoprotein (RNP) complexes that regulate certain protein translation involved in neuronal development and plasticity [7].

FMRP also contains a nuclear localization signal (NLS) and a nuclear export signal (NES) for shuttling certain mRNAs between the nucleus and cytoplasm [8, 9], albeit the function remains to be determined. As previous studies have shown that *FMR1* gene is inactivated by the over-expansion of CGG trinucleotide repeats (i.e., r(CGG) $\geq$200 copies) and the hypermethylation of the *FMR1* r(CGG) and promoter regions in 99 % of FXS patients [10], the pathological cause of FXS likely resides in certain interactions between r(CGG) over-expansion and *FMR1* hypermethylation. Conceivably, r(CGG)-derived miRNAs may play a key role in this gene silencing mechanism.

Since RNA is a highly degradable material, research of the miRNA-associated FXS mechanisms requires the use of fresh samples isolated from either patient's brain biopsies or comparable animal models. Due to lack of fresh human samples, almost all current studies adopt animal models instead. Zebrafish (*Danio rerio*) has been served as an excellent model for studying human mental disorders, including FXS and autism [11].

They possess three *FMR1*-related familial genes, *fmr1*, *fxr1*, and *fxr2*, which are orthologous to the human *FMR1*, *FXR1*, and *FXR2* genes, respectively [12]. The tissue expression patterns of these familial genes in zebrafish are broadly consistent with those of humans as well [12, 13]. To investigate the effects of r(CGG)-derived miRNA over-expression on brain development, we had found and sequenced a 450-nucleotide r(CGG) expansion motif located in and beyond the zebrafish *fmr1* 5′-untranslational region (5′-UTR), which contains approximately 50 copies of

r(CGG) similar to the numbers in normal human *FMR1* gene [3, 4]. In this r(CGG)-rich region, we further identified and isolated several r(CGG)-derived miRNAs, providing the first in vivo evidence of miRNA involvement in FXS pathogenesis [3, 4]. In view of all these genetic similarities between human and zebrafish, studies using this zebrafish FXS model not only have shed significant light on the pathological mechanisms of human FXS but also are useful for developing potential therapies for treating FXS-associated disorders.

Based on the current findings, miRNAs are involved in FXS during three developmental stages, including pre-blastocyst, post-blastocyst, and neural development, as illustrated in Fig. 7.1. Embryonic cells prior to the blastocyst (32–64-cell) stage are undifferentiated. This is because embryonic stem cell (ESC)-specific miRNAs, in particular miR-302, induce the expression of ESC-specific transcription factors, such as Oct4, Sox2, and Nanog, to maintain the undifferentiated state of stem cells and inhibit developmental signals [14, 15]. In addition, miR-302 also targets the *FMR1* mRNA 3′-UTR nucleotide 2774–2789 region (accession number NM_002024) for translationally suppressing FMRP synthesis. Such miR-302-mediated FMRP suppression is

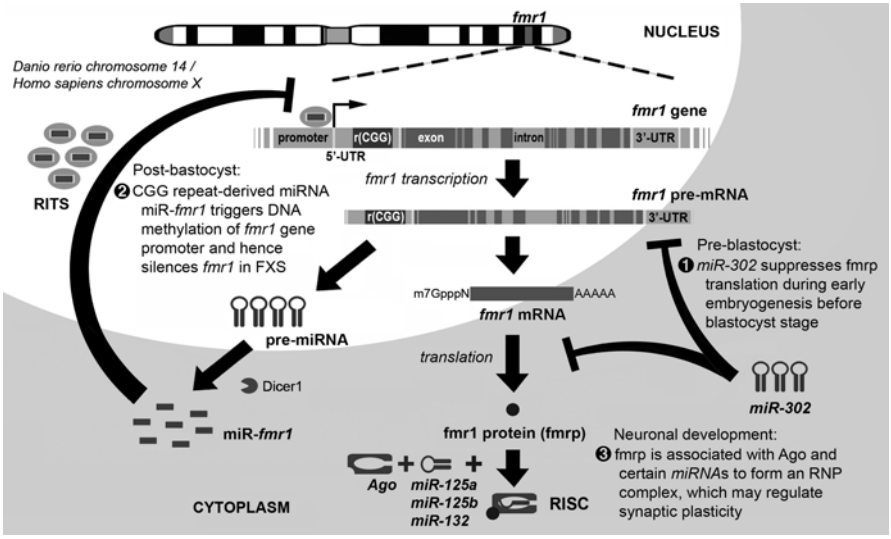

Fig. 7.1 Functional roles of *FMR1*/FMRP-associated miRNAs in neural development. Three groups of miRNAs were identified to affect *FMR1* and FMRP expressions at different developmental stages: (1) Before the embryonic blastocyst stage, miR-302 suppresses *FMR1* mRNA translation to maintain the ESC status and inhibit neural development. (2) After blastocyst, the expression of *FMR1* mRNA generates r(CGG)-derived miR-fmr1 miRNAs. In FXS, the overexpression and over-accumulation of miR-fmr1s in the neuron nuclei trigger DNA hypermethylation and hence inactivate *FMR1* transcription, the most prevalent event in FXS. (3) During neural development, miR-125a, miR-125b, and miR-132 may interact with FMRP to regulate the translation of certain neural proteins required for maintaining normal synaptic connection and plasticity. In FXS, the loss of FMRP may abolish these miRNA-mediated regulations and thus leads to the pathologies of FXS-related disorders, such as mental retardation and autism

a normal developmental event during the pre-blastocyst stage in order to maintain the pluripotency of early ESCs.

After blastocyst, most of the ESC-specific miRNAs are downregulated and then ectodermic differentiation begins. At this post-blastocyst stage, *FMR1* expression is escalated and its transcripts are quickly accumulated. As a result, the processes of *FMR1* mRNA maturation generate r(CGG)-derived miRNAs. In normal individuals, the level of r(CGG)-derived miRNAs is not sufficient to affect *FMR1* gene expression; yet, the high expansion of r(CGG) in FXS causes over-expression and over-accumulation of these r(CGG)-derived miRNAs and hence leads to the inhibition of *FMR1* and its protein FMRP expression. Since FMRP is responsible for regulating miR-125- and miR-132-mediated protein translation required for maintaining neuronal connectivity and synaptic plasticity [16, 17], the deficiency of FMRP disrupts these miRNA-mediated regulations and consequently results in premature neuronal development. In view of this established disease model, we will further discuss the functional roles of r(CGG)-derived miRNA, miR-125 and miR-132 in FXS pathogenesis.

Identification of r(CGG)-Derived miRNAs in a Zebrafish FXS Model

Human and zebrafish brains share many genetic and structural similarities [11–13]. As it is impossible to directly experiment on living human brain tissues, the current studies of r(CGG)-derived miRNAs are mainly based on a transgenic zebrafish FXS model. To mimic human FXS, an r(CGG)-rich DNA motif isolated from the zebrafish *fmr1* 5′-UTR r(CGG) expansion region (accession number NW001511047 from the 124001st to 124121st nucleotide) is transgenically overexpressed with high titer retroviral delivery into zebrafish brains [3, 4].

The over-expression of this r(CGG)-rich motif is mediated by an isolated gamma-aminobutyric acid receptor *ßZ2* (*GABAR2*) promoter and hence only occurs in the GABAergic neurons of cortex, hippocampus, and cerebellum [4, 5]. Since previous studies have established that *GABAR2* and *FMR1* genes are co-expressed in the GABAergic neurons [18, 19], the zebrafish FXS model so obtained can specifically reflect the damages of r(CGG)-derived miRNAs in the *FMR1*-positive neurons and subsequently prevents any potential off-target effect in other kinds of neuronal cells. Furthermore, by adjusting the multiplicity of retroviral infection (MOI), we can control the expression levels of r(CGG)-derived miRNAs to a certain amount sufficient to trigger the transcriptional suppression of *FMR1*, resembling the same etiological mechanism of human FXS [4, 5].

As depicted in Fig. 7.2a, wild-type zebrafish contains approximately 35–50 copies of CGG repeats in the *fmr1* 5′-UTR, whereas the r(CGG) number in the *FMR1* of human FXS is over 200, which conceivably may generate more than four times of r(CGG)-derived miRNAs.

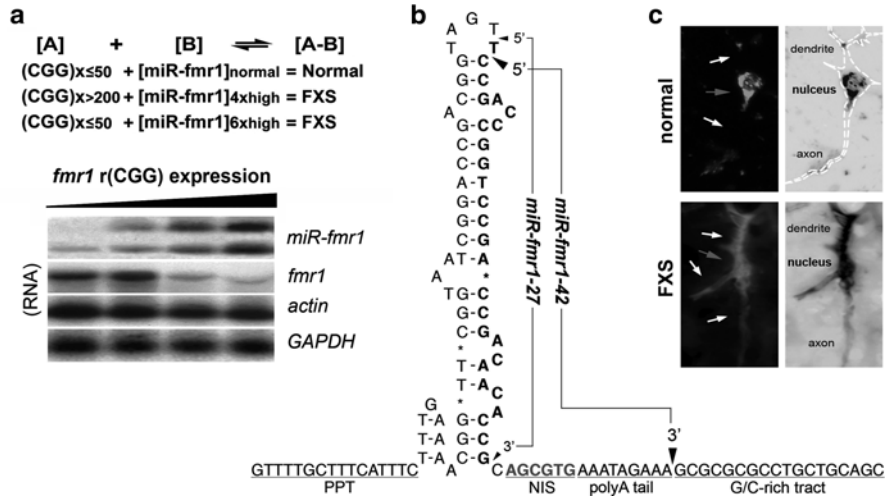

Fig. 7.2 Model of the transgenic r(CGG)-overexpressing zebrafish resembling human FXS. Normal zebrafish *fmr1* 5′-UTR region contains 35–50 r(CGG) copies, whereas the estimated r(CGG) number in human FXS *FMR1* is ≥200. In order to mimic the expression levels of human miR-fmr1s in FXS, the *fmr1* r(CGG) region was overexpressed ≥6-folds, leading to pathological results similar to human FXS. (**a**) Dose-dependent correlation between r(CGG)-derived miR-fmr1 expression and transcriptional *fmr1* suppression, determined by northern blotting ($p < 0.01$). The levels of miR-fmr1 expression and *fmr1* knockdown were proportional to the expression rates of r(CGG) expression. (**b**) Sequence and structural features of the r(CGG)-derived miR-fmr1 precursor. From the 5′ to 3′ end, it contains several key motifs, such as a poly-pyrimidine tract (PPT), an r(CGG)-rich hairpin-like pre-miRNA structure, a NIS, a short poly(A) tail, and sometimes a G/C-rich tract. Two miRNA isoforms had been identified as miR-fmr1-27 and miR-fmr1-42. (**c**) In situ hybridization with an anti-miR-fmr1-27 probe showed the different miR-fmr1 expression patterns between normal (wild-type) and FXS pallium–neocortical neurons

To create a condition similar to the r(CGG) over-expression in human FXS, we have used retroviral delivery to introduce a *GABAR2*-mediated r(CGG)-expressing *red-shifted green fluorescent protein* (*RGFP*) transgene into zebrafish [4, 5]. An r(CGG)-rich DNA motif isolated from the zebrafish *fmr1* 5′-UTR is manually placed in the 5′-UTR of the *RGFP* gene. After increasing the r(CGG) expression over sixfolds of the normal level in zebrafish, FXS-like disorders start to be observed in the targeted GABAergic neurons, indicating the minimal threshold of the r(CGG) over-expression levels required for inducing *fmr1* gene suppression. Accordingly, since the diseased human *FMR1* possesses over 200 copies of r(CGG), this threshold may be actually lower than sixfolds in human FXS. Following the elevation of r(CGG) over-expression, we have further identified the presence of two r(CGG)-derived miRNAs, namely miR-fmr1s, both of which are increased corresponding to the levels of r(CGG) expression. Meanwhile, the *fmr1* gene transcription is decreased in response to the increase of miR-fmr1 expression, suggesting that the expressed r(CGG) RNA transcripts can be processed into miR-fmr1s and then result in suppressing the *fmr1* gene transcription.

The sequences of these two r(CGG)-derived miR-fmr1s have been confirmed to be miR-fmr1-27 and miR-fmr1-42, respectively, as shown in Fig. 7.2b. They share a 26-nucleotide overlapping sequence in their 5′-end and are derived from the *fmr1* 5′-UTR r(CGG) expansion region approximately 65-nucleotide upstream of the translational start codon (accession number NM_152963). Both miR-fmr1s contain the same CCG-rich seed sequence for targeting the r(CGG) expansion region of the *fmr1* gene.

Notably, the miR-fmr1-42 further possesses three unique structures in its precursor microRNA (pre-miRNA) sequence, including (1) multiple matched CGG-CCG base pairs in the stem arm of the hairpin-like pre-miRNA, (2) a nuclear import signal (NIS) motif located in the 3-end of the hairpin structure, and (3) multiple CCG-rich DNA binding motifs in the mature miR-fmr1-42 sequence. Based on these structures, it is conceivable that the NIS motif may allow the entry of mature miR-fmr1-42 into the cell nucleus via a certain unidentified mechanism, while the multiple CCG-rich DNA binding motifs are involved in transcriptional suppression of the *fmr1* gene. In addition, the NIS motif is often flanked with a short poly-A tail, which may facilitate the decay of miR-fmr1-42 and hence prevents the miRNA accumulation in the normal neurons.

The expression patterns of both miR-fmr1 isoforms have been identified in the wild-type zebrafish brain, particularly located in the lateral pallium-neocortical and cerebellar neurons, as determined by fluorescent in situ hybridization (FISH) staining with a specific locked nucleic acid (LNA) probe directed against the miR-fmr1-27 sequence (Fig. 7.3). As shown in Fig. 7.2c, the FISH staining also indicated that wild-type pallium neurons (equivalent of human hippocampal stratum radiatum) present a limited amount of miR-fmr1s in the cytoplasmic region surrounding the cell nucleus, whereas the r(CGG)-overexpressing FXS neurons exhibit abundant miR-fmr1 expression and accumulation in all over the dendrite, soma, and nucleus areas. Further analyses using northern blotting of the two miR-fmr1s isolated from either cytoplasm or nuclei of the pallium neurons revealed that miR-fmr1-42 is the only miRNA accumulated in the nuclei of the FXS neurons [4, 5].

After deleting the NIS motif, the accumulation of miR-fmr1-42 is dramatically reduced in the nucleus portion but found more in the cytoplasm, suggesting that NIS may be required for the transporting of miR-fmr1-42 from the cytoplasm to the nucleus [4].

Following the nuclear accumulation of miR-fmr1-42, a marked increase of epigenetic DNA methylation in the *fmr1* promoter and its upstream region has been detected by bisulfite PCR and sequencing assays [4]. It is also noted that these DNA hypermethylation events occur mostly in the CpG-rich binding sites of several *fmr1*-associated transcriptional cofactors, such as NRF1 (GCGCGC), SP1 (GC box), and USF1/USF2 (E box). As previous studies have reported that blocking the binding of these transcriptional cofactors may cause transcriptional inactivation of *FMR1* in human FXS [20], the currently established r(CGG)-overexpressing zebrafish model not only successfully reveals this pathological process but also provides significant insights into the molecular mechanism underlying miRNA-mediated *FMR1* inactivation.

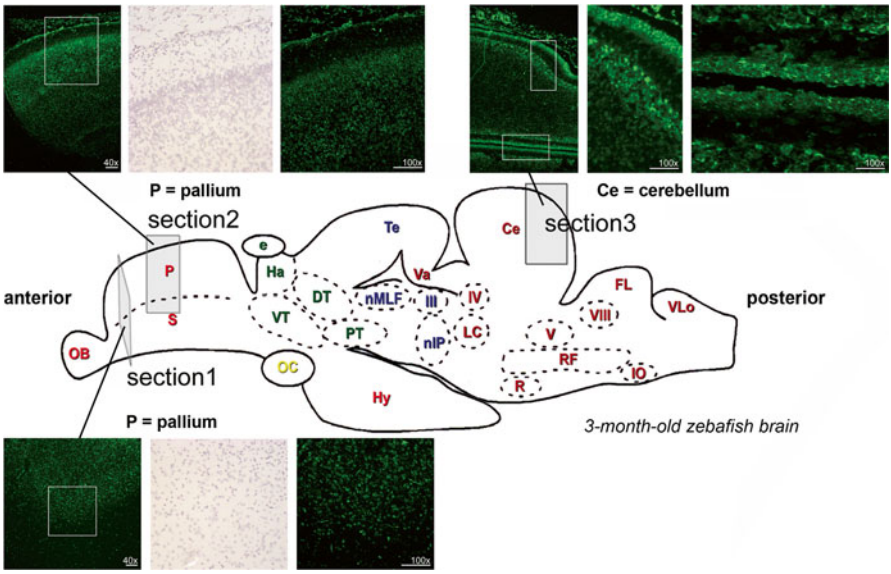

Fig. 7.3 Expression patterns of miR-fmr1s in the wild-type 3-month-old zebrafish brain. In situ hybridization assays showed the normal miR-fmr1 expression mainly in the lateral pallium (*section 1*), pallium–neocortical junction (*section 2*), and cerebellum (*section 3*)

Role of miR-fmr1 in the Etiological Mechanism of FXS

In view all of the above studies, Fig. 7.4 summarizes the etiological mechanism of FXS, in which excessive expression of r(CGG)-derived miRNAs, such as miR-fmr1s, causes miRNA accumulation in the neuron nuclei and then forms RNA-induced transcriptional silencing (RITS) complexes to trigger massive DNA methylation in the *FMR1* gene promoter, consequently leading to the inactivation of *FMR1* transcription. As in this zebrafish FXS model we only overexpressed one-third of the ~50 r(CGG) copies in wild-type *fmr1* and had already identified two miR-fmr1 isoforms, it is estimated that the ≥200 r(CGG) expansion in the diseased *FMR1* gene of human FXS may generate over 12 different kinds of r(CGG)-derived miRNA isoforms to inactivate *FMR1* transcription.

As a result, it will be very difficult to target these various miR-fmr1s for treatment. Also, how to demethylate the methylated *FMR1* gene promoter in FXS neurons is another problem. To overcome these problems, we need to understand the detail RITS compositions in order to prevent the assembly between RITS and miR-fmr1s; yet, till now the RITS components remain largely unknown.

To unveil the compositions of RITS, our studies have found that Dicer1 rather than Drosha is required for the r(CGG)-derived miRNA biogenesis, while Rad54l and MeCP2 may play crucial roles in the RITS assembly with miR-fmr1s [4, 5]. Using antisense morpholino oligonucleotides directed against certain key RNAi-

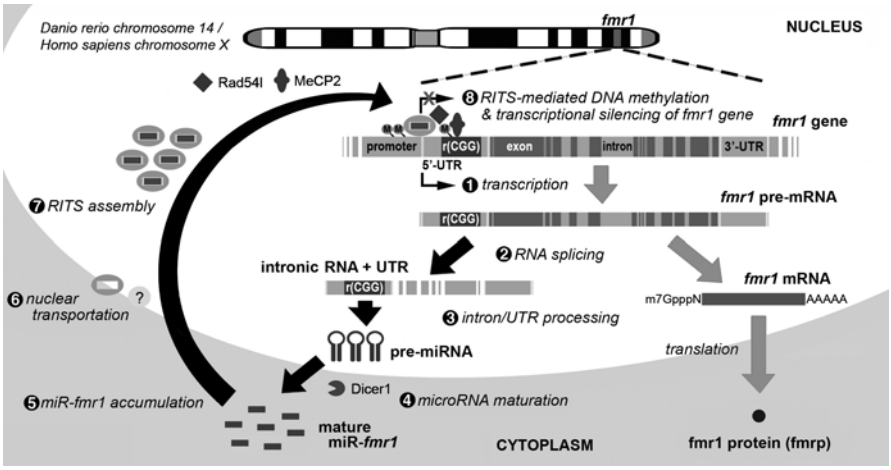

Fig. 7.4 Mechanism of miRNA-mediated FXS pathogenesis. Over-expansion of r(CGG) in the *FMR1* gene that encodes FMRP underlies FXS-associated disorders, such as mental retardation and autism. Repeats expanded over 200 copies (full mutation) lead to a complete loss of *FMR1* and FMRP expression. The pathological progression causing such FMRP deficiency includes eight steps: (1) Transcription of the *FMR1* gene starts after the embryonic blastocyst stage (i.e., day 7–10 human embryo or 10–12 h post-fertilization zebrafish). (2) RNA splicing and further processing excise the 5′-UTR r(CGG) expansion of *FMR1* mRNA into fragments. (3) The excised r(CGG) fragments are further processed into various repeat-associated miRNA precursors by certain ribonucleases. (4) These miRNA precursors are exported to cytoplasm and then cleaved into mature miR-fmr1s by RNaseIII Dicers. (5) Excessive miR-fmr1s are accumulated in cytoplasm around the cell nucleus. (6) miR-fmr1s with a NIS signal enter the cell nucleus. (7) Nuclear accumulation of miR-fmr1s induces the formation of RNA-induced transcriptional silencing (RITS) complexes with Rad54l and MeCP2 near the *FMR1* gene promoter. (8) RITS complexes trigger DNA hypermethylation and hence lead to transcriptional *FMR1* inactivation, the most prevalent event in over 99 % of the FXS patients

and CpG methylation-associated effector genes [4], we discovered that the mechanism of miR-fmr1-mediated gene silencing requires activities of Dicer 1 RNase, Rad54-like protein (Rad54l) and methyl-CpG binding protein 2 (MeCP2), but not Drosha RNase. Since the processing of intronic miRNAs has been known to bypass Drosha [21–23], the miR-fmr1 biogenesis may function through a similar process.

Moreover, our results also showed that both miR-fmr1 biogenesis and *fmr1* inactivation can be prevented by the morpholino-mediated knockdown of Dicer 1, while only the *fmr1* inactivation but not miR-fmr1 biogenesis is affected by the knockdown of either Rad54l or MeCP2. To this, further studies revealed that the *fmr1* gene transcription in FXS neurons is increasingly re-activated in response to the decrease of either Rad54l or MeCP2 expression, suggesting that both Rad54l and MeCP2 activities are likely required for the miRNA-mediated *fmr1* inactivation. As previous reports have manifested that both Rad54l and MeCP2 are involved in the CpG methylation of repetitive chromatin sequences in autism spectrum disorders [24], a similar mechanism may be reiterated to cause the miRNA-induced hypermethylation of the *FMR1* r(CGG) expansion and promoter regions in human FXS.

Pathologies of miR-fmr1-Induced FXS Disorders

The pathological outcomes of the currently established miR-fmr1-mediated FXS animal model are completely reminiscent of the neurodegenerative and cognitive impairments in human FXS disorders, including neuronal deformity, immature synapse formation, long dendritic spine shaping, diminishment of long-term potentiation (LTP), and augmentation of group 1 metabotropic glutamate receptor-dependent long-term depression (mGluR1-LTD).

Alterations of neurite growth and synaptic connectivity have been examined in the FXS zebrafish brains, resembling the exact pathological features of human FXS neurons (Fig. 7.5). Formation of long stripe neuronal dendrites is an important mark of human FXS. In fish lateral pallium (equivalent of human hippocampal stratum radiatum), wild-type neurons present normal neurite growth and branching dendrification, whereas miR-fmr1-affected FXS neurons exhibit long stripe dendrites and disconnected synapses similar to those found in the human FXS hippocampal–neocortical junction [3–5]. In particular, high density of long, immature dendritic spines is markedly increased, indicating failures in forming normal synaptic connections between these FXS-affected neurons (Fig. 7.5a).

In FXS patients, changes in spine shape are often linked to the absence of FMRP function [25]. FMRP is a translational inhibitor associated with local protein synthesis of certain mRNA species involved in neurite growth and synaptic connection, providing a crucial regulatory process for eliminating immature synapses and enhancing synaptic strength during normal brain development [26–28]. As a result, the miR-fmr1-mediated *FMR1* suppression causes FMRP deficiency and hence hinders synaptic strengthening through the formation of protein synthesis-dependent synaptic connections, consequently leading to a cascade of events that FXS is strongly implicated.

Impairment of synaptic plasticity is another major symptom of human FXS. Two types of synaptic plasticity are dependent on protein synthesis: LTP and long-term depression (LTD). LTP is a long-term increase in synaptic strength in response to high-frequency stimulation, whereas LTD is a long-lasting decrease in synaptic strength to below the normal baseline level after prolonged, low frequency stimulation. Both LTP and LTD underline the encoding activities of new declarative memories in brain [29]. LTP in hippocampus is a learning-associated form of synaptic plasticity that is highly involved in the shape change of dendritic spines [30].

It is also known that theta activity (3–8 Hz) is the major late-phase (protein synthesis-dependent) stimulation during the hippocampal encoding of long-term memory [29]. As shown in FXS zebrafish neurons (Fig. 7.5b), theta burst-stimulated synapses of the pallium–neocortical junction (equivalent of human hippocampal CA1–CA3) exhibit diminished LTP compared to that of the wild-type controls [4, 5]. This decreased LTP remains after the blockade of metabotropic GABAalpha receptor ($GABA_AR$)-dependent synaptic inhibition by picotoxin treatment, similar to the reported response of human FXS neurons [4]. In addition, a decrease of the input–output currents, measured at the peak amplitude of dendritic field excitatory

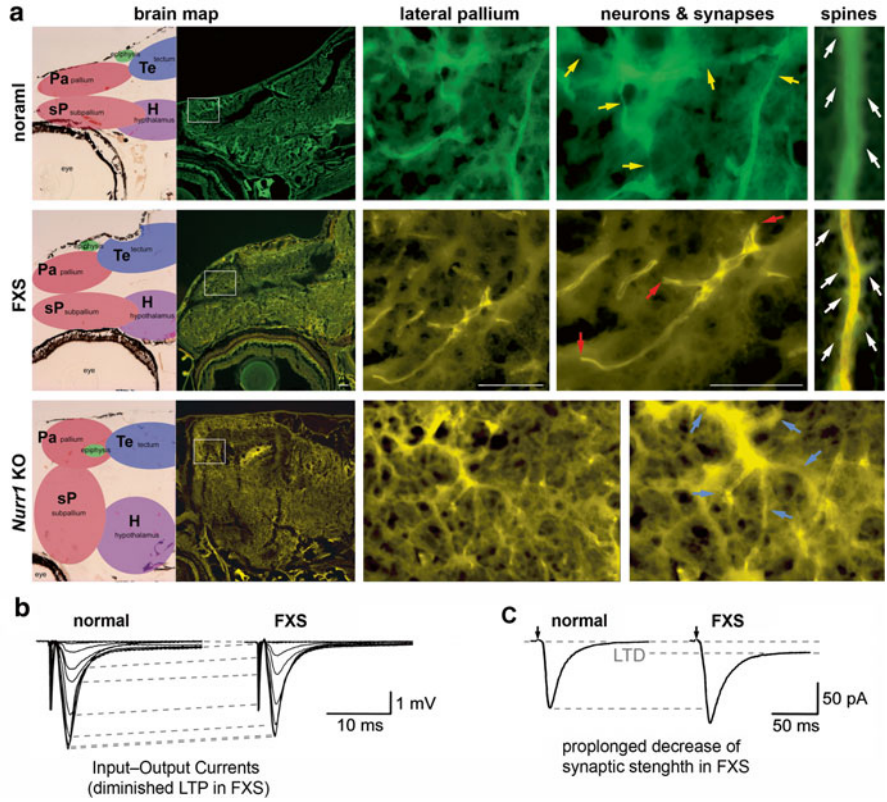

Fig. 7.5 Pathological alterations of FXS neurons. (**a**) Morphological changes of lateral pallium neurons in FXS (*middle row*) and *Nurr1*-knockdown (*Nurr1* KO; *bottom row*) zebrafish as compared to the wild-types (*top row*). Using fluorescent 3D-micrograph examination, three different kinds of neuronal connectivity were observed, including normal (wild-type), disrupted (FXS), tangled (*Nurr1* KO) neurite growth and synaptic circuit formation. *White arrows* indicated the formation of dendritic spines (most right panels). The *Nurr1* KO transgenics were generated by retroviral transfection of miR-739 into zebrafish, showing the distinctions between different miRNA functions. Abbreviations indicated: *Pa* pallium, *sP* subpallium, *Te* tectum, *H* hypothalamus. (**b**) Standard curves of synaptic LTP responses in pallium slices isolated from FXS zebrafish. Synaptic input–output fEPSP curves were evoked by varying bipolar current intensities, from 5.0, 10.0, 15.0, 25.0, 45.0, 65.0, 100.0 to 155.0 μA (pulse duration 0.1 ms). Calibration: 1 mV, 10 ms. (**c**) Pulse-induced LTD curves were measured at 40 μA for 125 ms. Calibration: 50 pA, 50 ms

postsynaptic potentials (fEPSPs), occurs corresponding to the diminished LTP, indicating a lower excitatory membrane response in the FXS neurons [4]. Given that the excitability of LTP of GABAergic neurons is mediated by activation of mGluR1 [31], further studies of the connection between miRNA-mediated FMRP deficiency and mGluR1 activity may provided answers to the pathological mechanism underlying LTP diminishment in FXS.

Post-synaptic stimulation of mGluR1 has been reported to increase the synthesis of certain neural proteins that trigger internalization of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPAR), leading to a process crucial for LTD expression [32, 33]. FMRP, one of mGluR1-stimulated proteins, is responsible for quenching this LTD process [33]. In FXS, the loss of FMRP in hippocampal neurons fails to quench LTD and hence results in a marked decrease of the LTP excitability, as shown in Fig. 7.5c. Yet, this prolonged LTD remains after co-treatment of an mGluR agonist, 3,5-dihydroxyphenyglycine (DHPG), and an *N*-methyl-D-aspartate receptor (NMDAR) antagonist, D-2-amino-5-phosphonovalerate (D-APV), suggesting that it is not mediated by NMDAR [4, 5].

Further treatment of DHPG with anisomycin, a protein synthesis inhibitor, completely prevents the mGluR1-LTD augmentation in normal rather than FXS neurons [5], indicating that FMRP may regulate mGluR1-LTD through both protein synthesis-dependent and protein synthesis-independent mechanisms. Since FMRP functions to suppress the translation of certain proteins involved in neural development and synaptic plasticity [8, 9], its deficiency in FXS may lead to excessive accumulation of these FMRP-suppressed proteins which in turn augment the protein synthesis-dependent mGluR1-LTD. On the other hand, emerging studies also showed that FMRP is associated with certain neuron-specific miRNAs, which may contribute to the protein synthesis-independent LTD inhibition.

Roles of FMRP-Associated miRNAs in Neural Development

Recent studies have found several miRNA species associated with FMRP in maintenance of normal neuronal development and synaptic plasticity. Using microarray analyses of miRNAs co-precipitated with FMRP isolated from wild-type and *FMR1*-knockout mouse brains, 12 candidate miRNAs were identified to specifically interact with wild-type FMRP, including let-7c, miR-9, 100, 124, 125a, 125b, 127, 128, 132, 138, 143, and 219 [17]. Among them, only miR-125a, 125b, and 132 have known functions in relation to FXS [16, 17], while others remain under investigation.

Current studies showed that FMRP does not directly contact with miR-125a; yet, FMRP phosphorylation promotes the assembly of AGO2 and miR-125a into an RNA-induced silencing complex (RISC), which in turn suppresses post-synaptic density protein 95 (PSD-95) mRNA translation [16]. PSD-95 is a protein member of the membrane-associated guanylate kinase (MAGUK) family, which is almost exclusively located in the post-synaptic density of neurons and is involved in anchoring synaptic proteins [34, 35]. It directly or indirectly binds with neuroligin, NMDAR, AMPA receptors, and potassium channels to form a multimeric scaffold for their clustering and hence plays an important role in synaptic plasticity and the stabilization of synaptic changes during LTP [35, 36].

Activation of mGluR1 stimulates the translation of PSD-95 mRNA and leads to AMPAR internalization [37], a process important for LTD expression. Interestingly,

studies also found that mGluR1 signaling further triggers the dephosphorylation of FMRP to dissociate the AGO2–miR-125a RISC complex and thus prevents the inhibitory effect of FMRP on PSD-95 translation [16]. Based on this finding, it is conceivable that the loss of FMRP in FXS may cause PSD-95 over-expression and ultimately results in mGluR1-LTD augmentation.

FMRP was also recently reported to affect NMDAR signaling via a negatively regulatory mechanism involving miR-125b and miR-132 [17], albeit no alteration of NMDAR-mediated LTD found in human FXS. Studies showed that both miR-125b and miR-132 have inhibitory effects on dendritic spine morphology and synaptic physiology in mouse hippocampal neurons, while FMRP knockdown can alleviate these effects [17].

Further target screening analyses revealed that miR-125b and AGO1 form certain RISC assembly to specifically suppress NMDAR subunit NR2A mRNA translation [17]. This silencing effect of miR-125b on NR2A may be one of many reasons causing dendritic spine changes; nevertheless, how does it relate to the known FXS pathologies such as diminished LTP and prolonged mGluR1-LTD remains unclear. Since there is no prior report of such an NMDAR-LTD alteration in the established mouse or zebrafish FXS model, research in this new direction may need to depend on the development of a novel animal model, in which NMDAR-LTD is affected by the loss of FMRP. Alternatively, it is possible that this NMDAR-LTD alteration may be covered or compensated by some unknown mechanisms in the current FXS animal models. To solve these questions, further investigation is needed.

Research of miRNA involvement in FXS is still at its very early stage. Although there are many miRNAs associated with FMRP, the majority of them currently do not have a clear function in relation to FXS. Yet, in view of this vast number of FMRP-associated miRNAs, it is conceivable that studies in this direction may discover fruitful knowledge to understand the detail processes of FXS pathogenesis and, in the long run, may lead to the development of novel medical interventions for treating FXS-related disorders, such as mental retardation and autism. The future application is very promising.

Conclusion

In sum, FXS is a genetic disease caused by dysregulation of multiple miRNA functions, including r(CGG)-derived miR-fmr1s and neuron-specific miR-125a, 125b, 132, and maybe many more waiting to be discovered. Based on the current understanding, the onset of FXS occurs after the embryonic blastocyst stage when ectodermic differentiation starts to form primordial neurons. At this critical moment for neural development, the expression of *FMR1* and its protein FMRP functions to regulate the translation of certain neural proteins, so as to enhance the establishment of normal synaptic connection and plasticity. However, due to the mutated over-expansion of r(CGG) in FXS patients' *FMR1* 5′-UTR, the expression of such mutated *FMR1* also generates an excessive amount of r(CGG)-rich fragments,

which are further processed into various miRNA isoforms, namely miR-fmr1s, by RNaseIII Dicers and are gradually accumulated in the cytoplasm and nuclei of neuronal cells. Since many of these miR-fmr1s carry NIS motifs for entering the cell nuclei, their nuclear over-accumulation results in the formation of RITS complexes with Rad54l and MeCP2, which then cause DNA hypermethylation of the r(CGG) expansion and its adjacent promoter regions of the *FMR1* gene, leading to completely shutdown of *FMR1* and FMRP expression. Without FMRP regulation, mGluR1 signaling will trigger the synthesis of excessive neural proteins that cause AMPAR internalization, pre-mature synaptic plasticity, LTP diminishment, and prolonged LTD expression, a cascade of events strongly implicated in FXS. In addition, since FMRP interacts with miR-125a and miR-125b/132 to regulate the signaling pathways of mGluR1 and NMDAR, respectively, its deficiency may abolish these miRNA-mediated regulations and further aggravate the FXS pathologies. To clarify the involvement of these miRNAs in FXS, their functional roles are depicted in Fig. 7.1.

The development of possible therapy for FXS is still under investigation. There are three major difficulties in its therapeutic design: First, how to demethylate the highly methylated r(CGG) expansion and promoter regions in FXS patients' *FMR1* gene? Second, how to deliver the drug through the blood–brain barrier (BBB)? Last, how to limit the drug effect only in GABAergic neurons? Currently, there are a few chemical drugs capable of causing DNA demethylation; yet, none of them are safe for treating neurons. Alternatively, recent findings of reprogramming-related miRNAs, such as miR-302, may offer a new approach to solve these problems.

Previous studies have shown that miR-302 enhances somatic cell DNA demethylation and hence reprograms the somatic cells into an ESC-like state [14, 15]. Using retroviral deliver of an inducible miR-302 transgene into zebrafish FXS neurons, we have found that there is a narrow window of miR-302 concentrations capable of silencing MeCP2 expression to demethylate the *fmr1* promoter region but not strong enough to suppress *fmr1* mRNA translation. Due to the similarity between human *FMR1* and zebrafish *fmr1* genes, this miR-302-mediated partial reprogramming approach may be useful for developing a novel therapy treating FXS. Nevertheless, how to maintain the miR-302 expression within such a narrow dosage window in a specific group of brain neurons will be challenging.

The findings in zebrafish and mouse FXS models have signified a vast similarity between these animal models and human FXS, which may shed light on new therapeutic interventions. Furthermore, these animal models may provide significant insights into the mechanisms of microsatellite-like nucleotide repeats in brain development for understanding their functional effects on human intelligence quotient (IQ) and autism.

Given that there are many different microsatellite-like nucleotide repeats in the human genome, which may encode a variety of repeat-associated miRNAs (ramRNAs), the availability of these animal models is surely useful for studying the functional roles of these various ramRNAs in vivo as a forthcoming challenge.

References

1. Handa V, Saha T, Usdin K. The fragile X syndrome repeats form RNA hairpins that do not activate the interferon-inducible protein kinase, PKR, but are cut by Dicer. Nucleic Acids Res. 2003;31:6243–8.
2. Krol J, Fiszer A, Mykowska A, Sobczak K, de Mezer M, Krzyzosiak WJ. Ribonuclease dicer cleaves triplet repeat hairpins into shorter repeats that silence specific targets. Mol Cell. 2007;25:575–86.
3. Lin SL, Chang SJE, Ying SY. First *in vivo* evidence of microRNA-induced fragile X mental retardation syndrome. Mol Psychiatry. 2006;11:616–7.
4. Chang SJE, Chang-Lin S, Chang D, Ying SY, Lin SL. Repeat-associated microRNA triggers fragile X syndrome in zebrafish. Open Neuropsychopharmacol J. 2008;1:6–18.
5. Lin SL, Ying SY. Role of repeat-associated microRNAs (ramRNA) in fragile X syndrome (FXS). In: Ying SY, editor. Current perspectives in microRNAs. New York: Springer; 2008. p. 245–66.
6. Hagerman RJ, Staley LW, O'Conner R, Lugenbeel K, Nelson D, McLean SD, Taylor A. Learning-disabled males with a fragile X CGG expansion in the upper premutation size range. Pediatrics. 1996;97:122–6.
7. Jin P, Alisch RS, Warren ST. RNA and microRNAs in fragile X mental retardation. Nat Cell Biol. 2004;6:1048–53.
8. Eberhart DE, Malter HE, Feng Y, Warren ST. The fragile X mental retardation protein is a ribonucleoprotein containing both nuclear localization and nuclear export signals. Hum Mol Genet. 1996;5:1083–91.
9. Tamanini F, Van Unen L, Bakker C, Sacchi N, Galjaard H, Oostra BA, Hoogeveen AT. Oligomerization properties of fragile-X mental-retardation protein (FMRP) and the fragile-X-related proteins FXR1P and FXR2P. Biochem J. 1999;343(Pt 3):517–23.
10. Sutcliffe JS, Nelson DL, Zhang F, Pieretti M, Caskey CT, Saxe D, Warren ST. DNA methylation represses FMR1 transcription in fragile X syndrome. Hum Mol Genet. 1992;1:397–400.
11. Tropepe V, Sive HL. Can zebrafish be used as a model to study the neurodevelopmental causes of autism? Genes Brain Behav. 2003;2:268–81.
12. Tucker B, Richards R, Lardelli M. Expression of three zebrafish orthologs of human FMR1-related genes and their phylogenetic relationships. Dev Genes Evol. 2004;214:567–74.
13. van 't Padje S, Engels B, Blonden L, Severijnen LA, Verheijen F, Oostra BA, Willemsen R. Characterization of FMRP in zebrafish: evolutionary dynamics of the fmr1 gene. Dev Genes Evol. 2005;215:198–206.
14. Lin SL, Chang D, Lin CH, Ying SY, Leu D, Wu DTS. Regulation of somatic cell reprogramming through inducible mir-302 expression. Nucleic Acids Res. 2011;39:1054–65.
15. Lin SL. Deciphering the mechanism behind induced pluripotent stem cell generation. Stem Cells. 2011;29:1645–9.
16. Muddashetty RS, Nalavadi VC, Gross C, Yao X, Xing L, Laur O, Warren ST, Bassell GJ. Reversible inhibition of PSD-95 mRNA translation by miR-125a, FMRP phosphorylation, and mGluR signaling. Mol Cell. 2011;42:673–88.
17. Edbauer D, Neilson JR, Foster KA, Wang CF, Seeburg DP, Batterton MN, Tada T, Dolan BM, Sharp PA, Sheng M. Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. Neuron. 2010;65:373–84.
18. Lopez-Bendito G, Shigemoto R, Kulik A, Vida I, Fairen A, Lujan R. Distribution of metabotropic GABA receptor subunits GABAB1a/b and GABAB2 in the rat hippocampus during prenatal and postnatal development. Hippocampus. 2004;14:836–48.
19. Selby L, Zhang C, Sun QQ. Major defects in neocortical GABAergic inhibitory circuits in mice lacking the fragile X mental retardation protein. Neurosci Lett. 2007;412:227–32.
20. Kumari D, Gabrielian A, Wheeler D, Usdin K. The roles of Sp1, Sp3, USF1/USF2 and NRF-1 in the regulation and three-dimensional structure of the fragile X mental retardation gene promoter. Biochem J. 2005;386:297–303.

21. Lin SL, Chang D, Wu DY, Ying SY. A novel RNA splicing-mediated gene silencing mechanism potential for genome evolution. Biochem Biophys Res Commun. 2003;310:754–60.
22. Ruby JG, Jan CH, Bartel DP. Intronic microRNA precursors that bypass Drosha processing. Nature. 2007;448:83–6.
23. Lin SL, Kim H, Ying SY. Intron-mediated RNA interference and microRNA (miRNA). Front Biosci. 2008;13:2216–30.
24. Lopez-Rangel E, Lewis ME. Loud and clear evidence for gene silencing by epigenetic mechanisms in autism spectrum and related neurodevelopmental disorders. Clin Genet. 2006; 69:21–2.
25. Irwin SA, Patel B, Idupulapati M, Harris JB, Crisostomo RA, Larsen BP, Kooy F, Willems PJ, Cras P, Kozlowski PB, Swain RA, Weiler IJ, Greenough WT. Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: a quantitative examination. Am J Med Genet. 2001;98:161–7.
26. Brown V, Jin P, Ceman S, Darnell JC, O'Donnell WT, Tenenbaum SA, Jin X, Feng Y, Wilkinson KD, Keene JD, Darnell RB, Warren ST. Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell. 2001;107:477–87.
27. Li Z, Zhang Y, Ku L, Wilkinson KD, Warren ST, Feng Y. The fragile X mental retardation protein inhibits translation via interacting with mRNA. Nucleic Acids Res. 2001;29: 2276–83.
28. Jin P, Zarnescu DC, Zhang F, Pearson CE, Lucchesi JC, Moses K, Warren ST. RNA-mediated neurodegeneration caused by the fragile X premutation rCGG repeats in *Drosophila*. Neuron. 2003;39:739–47.
29. Axmacher N, Mormann F, Fernandez G, Flger CE, Fell J. Memory formation by neuronal synchronization. Brain Res Rev. 2006;52:170–82.
30. Patenaude C, Chapman CA, Bertrand S, Congar P, Lacaille JC. GABAB receptor- and metabotropic glutamate receptor-dependent cooperative long-term potentiation of rat hippocampal GABAA synaptic transmission. J Physiol. 2003;553:155–67.
31. Godfraind JM, Reyniers E, De Boulle K, D'Hooge R, De Deyn PP, Bakker CE, Oostra BA, Kooy RF, Willems PJ. Long-term potentiation in the hippocampus of fragile X knockout mice. Am J Med Genet. 1996;64:246–51.
32. Snyder EM, Philpot BD, Huber KM, Dong X, Fallon JR, Bear MF. Internalization of ionotropic glutamate receptors in response to mGluR activation. Nat Neurosci. 2001;4:1079–85.
33. Nosyreva ED, Huber KM. Metabotropic receptor-dependent long-term depression persists in the absence of protein synthesis in the mouse model of fragile X syndrome. J Neurophysiol. 2006;95:3291–5.
34. Hunt CA, Schenker LJ, Kennedy MB. PSD-95 is associated with the postsynaptic density and not with the presynaptic membrane at forebrain synapses. J Neurosci. 1996;16:1380–8.
35. Sheng M, Sala C. PDZ domains and the organization of supramolecular complexes. Annu Rev Neurosci. 2001;24:1–29.
36. Meyer D, Bonhoeffer T, Scheuss V. Balance and stability of synaptic structures during synaptic plasticity. Neuron. 2014;82:430–43.
37. Bhattacharyya S, Biou V, Xu W, Schluter O, Malenka RC. A critical role for PSD-95/AKAP interactions in endocytosis of synaptic AMPA receptors. Nat Neurosci. 2009;12:172–81.

Chapter 8
The Emerging Role of MitomiRs in the Pathophysiology of Human Disease

Filipe V. Duarte, Carlos M. Palmeira, and Anabela P. Rolo

Abstract microRNAs (miRNAs) are small, single-stranded noncoding RNA molecules involved in posttranscriptional control of gene expression of a wide number of genes. miRNAs align and bind especially to 3′UTR sequences of their target genes and initiate either mRNA degradation or translational repression, resulting in reduced protein levels. miRNAs are now recognized as major players in virtually every biological process. In recent years, the discovery of miRNAs has revolutionized the traditional view of gene expression and our understanding of miRNA biogenesis and function has thereby expanded. The discovery of mitochondrial-located miRNAs raises the issue of the molecular mechanism underlying their translocation from the nucleus to the mitochondria. Studies in different species indicate that it may exist a number of import pathways of nucleus-encoded RNAs to mitochondria, being the most of them largely ATP-dependent. Not only pre-miRNAs, but also mature miRNAs, are present in the mitochondria; these findings have also raised the possibility of mitochondrial miRNA synthesis. Some pre-miRNAs sequences seem to be processed in the mitochondria, giving origin to mature miRNAs, which could be immediately active on the mitochondrial transcripts or exported to the cytosol in order to interfere with genomic-derived mRNA. Thus, the mitochondrial-processed miRNAs are likely to contribute to some posttranscriptional regulation of gene expression related to the mitochondrial functions. Coming from their location, the mitochondria, some miRNAs are currently named as mitomiRs; it refers to those miRNAs that can localize in mitochondria, whether transcribed from the nuclear or, potentially, the mitochondrial genome. When their genomics was analyzed, a number of mitomiRs mapped the nuclear genome at loci relevant to mitochondrial functions or diseases. Current computational analyses, using different algorithms, drive scientists to argue that the mitochondrial genome can harbor sequences that could

F.V. Duarte
Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra 3004-504, Portugal

C.M. Palmeira • A.P. Rolo (✉)
Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra 3004-504, Portugal

Department of Life Sciences, University of Coimbra, Coimbra 3000-456, Portugal
e-mail: anpiro@ci.uc.pt

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_8

be a target for several mitomiRs. However, perhaps a more challenging topic concerning mitomiRs is whether the mitochondrial DNA can harbor miRNA sequences, indicating an involvement of mitochondria in small RNA-generating pathways.

The identification of populations of miRNAs in the mitochondria pushed scientists in the field to question its biological functions. It is established that miRNAs, originated in the nuclear genome, are exported to cytosol where they are processed and exert their function by inhibiting nuclear genome-derived mRNA. Actually it is also known that some miRNAs are imported into mitochondria where they interact with some mitochondrial genome-derived mRNA molecules. More strikingly, it has also come to light that mitochondrial genome (mtDNA) can originate some miRNA molecules that exert their function directly on mitochondrial transcripts. The links between miRNA deregulation and human disease have been reported in almost all medicine fields. Currently, great efforts are being invested in understanding the involvement of miRNA deregulation in disease and unlocking the mechanisms by which they act. This new field of investigation has revealed the tremendous potential of miRNAs as diagnostic or even as valuable therapeutic tools.

miRNAs have recently emerged as key regulators of metabolism. Metabolic syndrome is a systemic disorder that includes a spectrum of abnormalities associated with obesity and type II diabetes. Defects in mitochondrial function, namely related to oxidation of fatty acids, have been linked to diet-induced obesity and the development of insulin resistance in adipose tissue and skeletal muscle. Consistently, obese individuals have mitochondria with compromised bioenergetic capacity. Therefore, increasing interest is being given to the role of miRNAs on metabolic regulation, with relevance on mitochondria and the mechanisms purported for miRNA actions, particularly acting in mitochondria or in mitochondria-related pathways. The involvement of miRNAs in mitochondrial metabolism, mitochondrial oxidative phosphorylation (OXPHOS), electron transport chain (ETC) components, lipid metabolism, and metabolic disorders is becoming more and more comprehended, as well as miRNAs contribution for processes such as mitochondrial dynamics or apoptosis regulation and cancer.

Keywords Alzheimer's disease • Amyotrophic lateral sclerosis (ALS) • Cancer • Glycolysis • Huntingtin (HTT) • Huntington's disease • Metabolic disorders • microRNA (miRNA) • Mitochondria • Mitochondrial DNA (mtDNA) • Mitochondrial dynamics • Mitochondrial transcripts • Mitofusin • mitomiRs • Neurodegeneration • OXPHOS • Parkinson's disease

Introduction

MicroRNAs (miRNAs) are short, single-stranded, noncoding RNA molecules (19–23 nucleotides) that act by posttranscriptional modulation of protein-coding genes, through repressing messenger RNA (mRNA) translation or promoting their degradation (see Chap. 2 of the volume "microRNA: Basic Science" for a detailed discussion of miRNA machinery). Each miRNA can affect multiple target genes [1].

miRNAs are now recognized as major players in almost every biological process such as cell proliferation, apoptosis, differentiation, and organogenesis [1, 2]. In recent years, the discovery of miRNAs has revolutionized the traditional view of gene expression and our understanding of miRNA biogenesis and function has expanded. The links between miRNA deregulation and human disease have been reported in almost all medicine fields. Currently, great efforts are being invested in understanding the involvement of miRNA deregulation in disease and unlocking the mechanisms by which they act. This new field of investigation has revealed the tremendous potential of miRNAs as diagnostic or therapeutic tools.

Many studies have already deeply focused on the characterization of mitochondrial DNA genetics, also trying to understand its modulation by miRNAs [3]. Mitochondria of all eukaryotes are the sole organelles (except for chloroplasts in plants) that have a distinct genome made of a double-stranded, circular DNA molecule (mtDNA). mtDNA mutations have been found to be implicated in a wide variety of both physiological (e.g., heat production, reactive oxygen species (ROS) generation, apoptosis, cellular differentiation, and aging) and pathological phenotypes, including neurodegenerative diseases, diabetes, metabolic syndrome, and several cancers [4, 5]. Furthermore, there is an extensive network of bidirectional signals between nuclear and mitochondrial genomes. This regulatory crosstalk is crucial for the activity of the entire cellular machinery, basically by modulating mitochondrial biogenesis and metabolism through reciprocal mitochondrial-to-nucleus communication [6, 7].

Relatedly, recent and increasing amount of data from whole genome and transcriptome sequencing suggest that both nuclear- and mitochondrial-encoded small noncoding RNAs (sncRNAs), including short interfering RNAs (siRNAs), microRNAs (miRNAs) as well as long noncoding RNAs (lncRNAs), are responsible for the regulation of important signaling pathways involving mitochondria. They have been suggested to modulate the expression of mitochondrial proteins encoded by nuclear or mitochondrial genes, and also the regulation of mitochondrial structure, function, and dynamics [8, 9].

Mitochondria

For several years mitochondria have been subject of extensive studies intended to unravel the role of these organelles in cellular physiology. In this regard, the complexity of cell physiological coordination has been recapitulated in a complex and network of signals that intersects and integrates with the intensive mitochondrial activity. Indeed, besides the production of most of cellular energy, mitochondria are able to modulate many intracellular signaling pathways, which are crucial for the maintenance of cellular homeostasis. They are sites of multiple metabolic pathways, including β-oxidation of fatty acids, tricarboxylic acid and urea cycles, control intracellular Ca^{2+} metabolism and signaling, ion homeostasis, steroid hormone biosynthesis, regulate thermogenesis and settle the cell fate by integrating numerous death signals [10].

Mitochondria are cellular organelles delimited by two membranes that embrace about one-tenth of the cell's proteins. The mitochondrion consists of four main structures or compartments: two membranes, the intermembrane space, and the matrix within the inner membrane. The mitochondrial outer membrane (MOM) separates the cytosol from the intermembrane space. The MOM is responsible for interfacing with the cytosol and its interactions with cytoskeletal elements, which are important for the movement of mitochondria within a cell. This mobility is essential for the distribution of mitochondria during cell division and differentiation.

The mitochondrial inner membrane (MIM) separates the intermembrane space from the matrix. The folding of the MIM (cristae) serves to increase the surface area of this membrane. The MIM hosts the most important redox reactions converting the energy of nutrients into ATP. These reactions are catalyzed by the mitochondrial electron transport chain (ETC), which transports electrons from several substrates to oxygen, in the complex multistep process termed mitochondrial respiration. According to the chemiosmotic theory, mitochondrial respiration generates a transmembrane potential ($\Delta\Psi m$) across the inner membrane, which is used by ATP synthase to phosphorylate ADP. The MIM is normally impermeable to protons and other ions, and this solute barrier function of the MIM is critical for energy transduction. Permeabilization of the MIM dissipates $\Delta\Psi m$ and thereby uncouples the process of respiration from ATP synthase, halting mitochondrial ATP production [11].

In addition to the process of ATP formation, mitochondria are highly dynamic organelles that have been implicated in the regulation of a great and increasing number of physiological processes. Mitochondrial function is a key to cell life and death, and the deregulation of mitochondrial metabolism is critical to the pathogenesis of several diseases. Cells need energy not only to support their vital functions but also to die gracefully, through programmed cell death, or apoptosis [11].

Furthermore, mitochondrial regulation is also present beyond cell death mechanisms. Indeed, besides oxidative ATP production, mitochondria assume other functions such as heme synthesis, β-oxidation of free fatty acids, metabolism of certain amino acids, production of free radical species, formation and export of Fe/S clusters, iron metabolism, and play a crucial role in calcium homeostasis [12]. Still, the regulatory roles of mitochondria over normal physiology include the transduction pathway that underlies the secretion of insulin in response to glucose by β-cells.

The physiological "uncoupling" of mitochondria also plays a central role as a heat-generating mechanism in non-shivering thermogenesis in young mammals. It has also been suggested that the production of free radical species by mitochondria may play a key role as a signaling mechanism, for example, in the regulation of ion-channel activities and also in initiating cytoprotective mechanisms in stressed cells [13].

Mitochondrial OXPHOS produces more than 95 % of a cell's energy in the form of ATP under normal physiological conditions. This process involves five different protein complexes, Complex I–V. The respiratory chain or electron transport chain (ETC; Complexes I–IV) in the MIM is associated with electron transfer components—coenzyme Q and cytochrome c. In the matrix, pyruvate oxidation, β-oxidation of fatty acids, and the TCA cycle pathways are associated.

The total number of polypeptides involved directly in OXPHOS is 91 when cytochrome *c* is included. Of these proteins, some are nuclear-encoded, and some are mitochondrial-encoded in the mtDNA [13]. Because of its limited coding capacity mtDNA relies on nuclear genes for structural components and biological functions. Besides, nuclear-encoded genes also regulate mitochondrial transcription, translation, and mtDNA replication, thus the precise cooperation of nuclear and mtDNA expression is essential to regulate OXPHOS capacity in response to different physiological demands and disease states [14, 15].

Dysregulated mtDNA expression has been associated with human mitochondrial diseases and is also observed in normal aging process [16, 17]. The overall process of oxidative phosphorylation (OXPHOS) is tightly controlled by transcriptional regulation at the level of DNA, translational effects via RNA levels and stability, by substrate feedback inhibition, and by posttranslational modifications, including phosphorylation and acetylation. Inefficient electron transfer through complexes I–IV causes human disease in part because of loss of energy metabolism but also because insults to the various enzymes (particularly Complexes I, II, and III) induce production of toxic ROS. Defects of complex V are also a cause of mitochondrial dysfunction [18]. It has also been reported that the deterioration of mitochondrial function underlies common metabolic-related diseases [19, 20], and several studies have identified compromised oxidative metabolism, altered mitochondrial structure and dynamics, and impaired biogenesis and gene expression in insulin resistance or type 2 diabetes (T2DM) models [21, 22].

Mitochondria also play a pivotal role in the process of cell death. It has been generally accepted that cells die by necrosis when ATP is not sufficient, or they die by the process of apoptosis when sufficient ATP is available. Apoptosis (programed cells death) is a process coordinated by a family of proteases—the caspases, which participate in the molecular control of apoptosis as triggers of cell death and as regulatory elements within this process. Excessive accumulation of Ca^{2+} leads to the formation of ROS and to opening of the mitochondrial permeability transition pore (mPTP), which depolarizes the mitochondria and leads to mitochondrial swelling.

This may also provide a mechanism for the release of cytochrome *c* from the intermembrane space into the cytoplasm. Cytochrome *c* normally functions as a part of the respiratory chain, but when released into the cytosol it becomes a critical component of the apoptosis execution machinery, where it activates caspases and causes apoptotic cell death. Apoptosis may be triggered by extracellular signals (extrinsic pathway) or by intracellular processes (intrinsic pathway) [23]. An increased mitochondrial formation of ROS triggers the intrinsic pathway by opening permeability transmission pores with increased permeability of the outer mitochondrial membrane.

Mitochondrial shape is very heterogeneous, ranging from small spheres to interconnected tubules. During cell life, mitochondria undergo continuous cycles of fusion and fission. Therefore, mitochondria are now recognized as highly dynamic organelles that move throughout a cell and constantly fuse and divide [24]. Studies over the last several years indicate that these membrane-remodeling processes promote homogenization of the mitochondrial population by content mixing and

thereby preserve mitochondrial function. It was reported, in pancreatic β-cells, that fusion and fission events are paired. Fusion triggers fission, but fission has no effect on the following fusion event [25]. Research on mitochondrial fusion and fission (collectively termed mitochondrial dynamics) gained much attention in recent years, as it is important for our understanding of many biological processes, including the maintenance of mitochondrial functions, apoptosis, and aging [26].

miRNAs in Mitochondria

It has been described that a subset of miRNAs, highly conserved noncoding RNAs with regulatory function, play a critical role in regulating both mitochondrial function and also the DNA methylation machinery [8, 27]. Among them, several examples of particular interest concern to miRNAs that affect mitochondrial function and mitochondrial metabolism in cancer cells, resulting in tumor suppression by inducing metabolic reprogramming [9].

Recent indications highlight the existence of a regulatory link between the expression of some miRNAs, mitochondria, and several disease phenotypes. Indeed, data from whole genome and transcriptome sequencing suggested that a large number of sncRNAs including siRNAs, miRNAs, and lncRNAs are responsible of the regulation of important signaling pathways in mitochondria [28]. As an example, 15 nuclear-encoded miRNAs, identified in mitochondria isolated from adult rat livers, appeared to be involved in the expression of genes associated with apoptosis, cell proliferation, and differentiation [29]. Despite several nuclear-encoded regulatory RNAs targeting to mitochondria, there have also been identified some stable and abundant mitochondrial lncRNAs transcribed from the regions of the mitochondrial genome complementary to ND5, ND6, and Cyt *b* genes [30]. Moreover, deep RNA sequencing studies carried out in human cardiac tissues revealed a high relative abundance of lncRNAs encoded by mitochondrial genome [31].

Regarding mitochondrial small noncoding RNAs (ncRNAs), there have also been found some ncRNAs mapping within the mitochondrial D-loop region others transcribed in antisense orientation ND4 and ND6 mRNAs [32]. Additionally, there are several other recent reports showing that both human and mouse mtDNA encode for thousands of small RNAs, which are mainly derived from the sense transcripts of the mitochondrial genes [33].

In the last years, it was reported that pre-miRNAs as well as mature miRNAs are present in the mitochondria, and these findings have also raised the possibility of mitochondrial miRNA synthesis [34, 35]. It was suggested that some pre-miRNAs sequences seem to be processed in the mitochondria and may be to synthesize mature miRNAs, which could be immediately active on the mitochondrial transcripts or exported in the cytosol in order to interfere with genomic mRNA. Thus, the mitochondrial-processed miRNAs are likely to contribute to some posttranscriptional regulation of gene expression related to the mitochondrial functions [36]. Some miRNA molecules were already predicted from the mitochondrial genome by bioinformatics analysis. More interestingly, a recent report has already experimen-

tally shown the accumulation of six miRNAs in human mitochondria [37]. The presence of these miRNAs was confirmed in mitochondria from human skeletal muscle; further tan that, luciferase assay was used to confirm that MT-RNR2 gene was the potential target of hsa-miR-mit3 and hsa-miR-mit4.

The discovery of mitochondrial-located miRNAs (mitomiRs) raises the issue of the molecular mechanism underlying their translocation from the nucleus to the mitochondria. Studies in different species indicate that it may exist a number of import pathways of nucleus-encoded RNAs to mitochondria, being the most of them largely ATP-dependent. Nonetheless, the molecular mechanisms of mitochondrial RNA import seem to vary largely in a species-specific way [34].

Coming from their location, the mitochondria, some miRNAs are currently named as mitomiRs; it refers to those miRNAs that can localize in mitochondria whether transcribed from the nuclear or, potentially, the mitochondrial genome. When their genomics was analyzed, a number of mitomiRs mapped the nuclear genome at loci relevant to mitochondrial functions or diseases [34]. Moreover, the set of mitomiRs may vary depending on the cell type. Indeed, a targeted approach was already used by customizing tissue-specific miRNA microarrays, which led to the identification of three rat cardiac-specific mitomiRs, namely miR-181c, miR-1192, and miR-883 [38].

The identification of populations of miRNAs in the mitochondria pushed scientists in the field to question its biological functions. It is established that miRNAs, originated in the nuclear genome, are exported to cytosol where they are processed and exert their function by inhibiting nuclear genome-derived mRNA. Actually it is also known that some miRNAs are imported into mitochondria where they interact with some mitochondrial genome-derived mRNA molecules. More strikingly, it has also come to light that mitochondrial genome (mtDNA) can originate some miRNA molecules that exert their function directly on mitochondrial transcripts [8, 34, 39].

Actually, apart from the presence in the cytosol of miRNA regulating mRNAs that encode proteins involved in mitochondria-related activities, a small number of miRNAs were reported to be present within the mitochondrial matrix itself [29, 40], with revealed apoptosis/cell-death and cell-cycle/cell-division as among the most significant processes targeted, but only one possible interaction with mtDNA-derived mRNA (between miR-130a and cytochrome c oxidase III). Moreover, unique sets of mitochondrial miRNAs, or mitomiRs, were already described to be present in human skeletal muscle and HeLa cells [41, 42].

The mitomiRs were found to have thermodynamic features and size distinct from canonical miRNAs and to be expressed almost entirely from nuclear genes in loci relevant to mitochondrial function. MitomiRs appeared to lack preferential targeting of nuclear-encoded mitochondrial genes, when compared with a set of cytosolic miRNAs, but most were predicted by RNA22, RegRNA, miRWalk, or TargetScan algorithms to target multiple mtDNA sites (including many within all the mtDNA-encoded protein genes). Interestingly, human mtDNA also seems to harbor mitomiR sequences (namely, miR-1974, miR-1977, and miR-1978), but it remains to be ascertained whether miRNAs are actually transcribed from these mitochondrial genes [41]. The general overview of miRNA origin and subcellular site of action is depicted in Fig. 8.1.

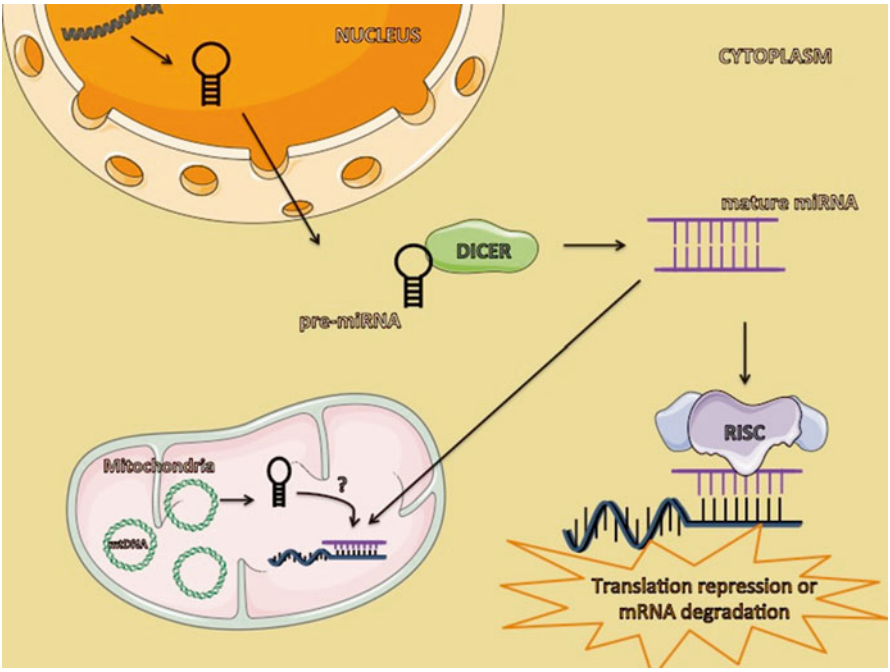

Fig. 8.1 Depiction of miRNA origin and site of action in the cell. Most of the known miRNAs are originated in the nucleus, from the nuclear genome, and then exported to cytosol where they are processed by DICER and exert their function when assembled in RISC complex, by inhibiting nuclear genome-derived mRNA. Currently it is also known that some miRNAs are further imported into mitochondria where they interact with some mitochondrial genome-derived mRNA molecules. More strikingly, it has also come to light that mitochondrial genome (mtDNA) can originate some miRNA molecules that exert their function directly on mitochondrial transcripts, although this pathway is still poorly understood

In mammalian cells, miRNAs are involved not only in disease phenotypes progress, but also in physiological mechanisms, such as switch of energy source during muscle differentiation or cellular differentiation and reprogramming. Interestingly, miRNA machinery also plays a role in the initiation of mitochondrial translation. A recent study has pointed out a role of miR-1 in enhancing protein synthesis and ATP production in the mitochondria of differentiated muscle cells, suggesting miR-1 as a coordinator of the regulatory networks in both the cytoplasm and the mitochondria during muscle differentiation [43]. microRNAs were already known to mediate translational repression and mRNA degradation in the cytoplasm. However, and despite the fact that several miRNAs have also been detected in membrane-compartmentalized organelles such as mitochondria, its functional meaning is not very clearly elucidated. In the muscle, it is already described that miR-1, a microRNA specifically induced during myogenesis, efficiently enters the mitochondria where it unexpectedly stimulates, rather than represses, the translation of specific mitochondrial genome-encoded transcripts.

These findings reveal a positive regulatory function of miRNAs in mitochondria, and suggest a new layer of crosstalk between nuclear and mitochondrial genomes in eukaryotic cells.

miRNAs in Mitochondrial Dynamics

Mitochondria are highly dynamic organelles that possess two opposing activities. They divide and fuse constantly, and the balance between mitochondrial fission and fusion affects the morphology of mitochondria, which dynamics and turnover are crucial for cellular homeostasis and differentiation [44]. Mitochondria form dynamic networks that are necessary for the maintenance of the organelle fidelity [45]. Various proteins participate in the regulation of mitochondrial dynamics, and a deregulation of mitochondrial dynamics is not only related to deregulation of mitochondrial function, but also closely correlated with apoptosis and several diseases.

miRNAs have emerged as an important class of posttranscriptional regulators of gene expression in virtually all fundamental cellular pathways, including mitochondria-associated signaling pathways. It is more than clear that their abnormal expression may results in pathophysiological processes in the cell and, consequently, in the onset of many diseases. It has been found that some miRNAs affect mitochondrial dynamics, thus impinging on the dynamic mitochondrial balance. miR-499 and miR-30 regulate the mitochondrial fission machinery by directly targeting DRP1 [46, 47], miR-19b negatively regulates mitochondrial fusion by downregulating mitofusin 1 (Mfn1) gene [48], and miR-106b is responsible for mitochondrial dysfunction by targeting mitofusin 2 (Mfn2) [49].

A recent study has revealed that miR-27 acts as a novel factor regulating mitochondrial dynamics by suppressing the mitochondrial fission factor (MFF) expression [50]. miR-27 suppresses the association of MFF mRNA with polysomes via its 3′-untranslated region (UTR). Moreover, it was shown that ectopic expression of the miR-27 precursor resulted in mitochondrial fusion, thereby increasing the mitochondrial membrane potential as well as the mitochondrial ATP level. Thus, it is now recognized that miR-27 is involved in negatively regulating mitochondrial fission by directly targeting MFF. Several other reports have shown that mitochondrial fusion can inhibit cell death, whereas mitochondrial fission is involved in the promotion of apoptosis. Given this, it is conjectured that the increased expression of miRNAs targeting genes needed for mitochondrial fission, such as miR-27, may enhance mitochondrial fusion, thereby promoting tumor progression [50].

Another example involves miR-761, which is responsible for the downregulation of the mitochondrial fission factor; it can suppress mitochondrial fission and apoptosis by targeting MFF, demonstrating that miR-761 and MFF constitute an axis in the machinery of the mitochondrial network and apoptosis [51]. Additionally, miR-30 family members have been reported to regulate apoptosis by targeting the mitochondrial fission machinery, inhibiting mitochondrial fission by suppressing the expression of p53 and its downstream target Drp-1 [47].

More recently, miR-140 has been demonstrated to be upregulated upon apoptotic stimulation, causing a downregulation of Mfn1 in cardiomyocytes [52]. miR-140 has been hence reported to play a functional role in mitochondrial fission and apoptosis, and knockdown of miR-140 was able to reduce myocardial infarct sizes in an animal model, exerting its effect on mitochondrial fission and apoptosis through targeting Mfn1.

Mitochondrial fission and its modulation by miRNA has also been shown in some metabolic conditions; miR-484 downregulation may contribute to the pathogenesis of insulin resistance by targeting mitochondrial fission protein 1 (Fis1), which is needed for mitochondrial fission [53], as mitochondrial fission is increased in diabetes and contributes to circulating insulin levels [54].

The removal of damaged mitochondria by autophagy, a process referred to as mitophagy, is critical for maintaining proper cellular functions. Mitophagy regulates the number of mitochondria to match the metabolic or developmental demands [55, 56], and is also a part of the quality control based on the removal of impaired mitochondria [44, 45]. Recent studies have suggested a role for miRNAs in autophagy, including miR-101 [57–59], miR-204 [59, 60], and miR-30a [61–63]. miRNAs target transcripts of autophagy-related proteins, thereby suppressing their function in autophagy with pathological consequences [63–65]. For example, downregulation of miR-34b/c is an early event in Parkinson's disease [66–68]. In some tumors, the level of miR-21 is increased, and it suppresses the expression of PTEN, which regulates the mitophagy-associated PINK1 [69, 70]. Mitophagy has been characterized as a HIF-dependent mechanism [55, 71]. Furthermore, hypoxic repression of the mitochondrial function by miR-210 and ISCU1/2 may trigger mitophagy.

In the last years, miR-494, whose location is predominantly mitochondrial, has been found to modulate mitochondrial biogenesis by downregulating the mitochondrial transcriptional factor A (TFAM) and the nuclear transcription factor Forkhead box J3 (FOXJ3) during myocyte differentiation and skeletal muscle adaptation to physical exercise [70, 72].

Mitochondrial miRNAs in Cancer

The traditional hallmarks of cancer, as defined and subsequently reviewed by Hanahan and Weinberg [36, 57, 59], comprise six biological capabilities acquired during the multistep development of human tumors. These six traditional cancer hallmarks include resisting cell death, inducing angiogenesis, sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, and enabling replicative immortality. Importantly, the same authors have recently added two novel hallmarks to the traditional ones, i.e., the reprogramming of the energy metabolism of the cell and evading immune destruction which provides a certain basis for a change of our perception of typical tumor characteristics [10, 59].

Under aerobic conditions, the normal cells generate ATP primarily in mitochondrial OXPHOS process, which utilizes products of glycolysis and Krebs cycle.

Under anaerobic conditions, relative little pyruvate, the end product of glycolysis, is directed to the Krebs cycle and it is converted to lactate instead. Nevertheless, this metabolic conversion of glucose appears to be energetically disadvantageous. Interestingly, it was observed that many cancer cells prefer glycolysis instead of OXPHOS even in the presence of sufficient amount of oxygen. This anomalous energy metabolism is known as the *Warburg effect* [36, 61, 63]. Although the exact causes and functional consequences of this metabolic switch remain to be elucidated, there is a growing consensus that the Warburg effect is not an inconsequential by-product of carcinogenesis, but is vital for cancer cells to maintain their proliferative potential, and is driven by various factors, including miRNAs [63, 64, 73].

The phenomenon of hypoxia has already been related to altered miRNA expression, identifying specific hypoxia-regulated miRNAs (HRMs) that play an important role in cell survival in low oxygen environment [74]. One of the miRNAs found to be consistently upregulated in normal and transformed cells during hypoxia is miR-210 [75], highlighting a role of miR-210 in cell adaptive response to hypoxia. In tumor tissues such as breast cancer and head and neck cancers, miR-210 expression levels have been demonstrated to be correlated with hypoxia gene signatures, which suggested a direct connection between miR-210 expression and hypoxia in cancer [76, 77]. The master HRM miR-210 has been investigated intensively, displaying a variety of functionally important targets involved in cell cycle regulation, cell survival, differentiation, angiogenesis, and metabolism [75].

Under hypoxic conditions, cell metabolism shifts from mitochondrial OXPHOS to glycolysis. HIF-1 plays a critical role in this effect, by upregulating the expression of most glycolytic enzymes as well as pyruvate dehydrogenase kinase, while downregulating mitochondrial respiration. The regulation of mitochondrial metabolism during hypoxia by miR-210 has already been addressed, demonstrating that miR-210 directly targets iron-sulfur cluster assembly proteins (ISCU1/2) and decreases the activity of iron-sulfur proteins controlling mitochondrial metabolism, including complex I and aconitase [78]; this results in decreased OXPHOS.

Subsequent studies investigating the role of miR-210 in modulating mitochondrial function have also revealed COX10, SDHD, and NDUFA4 as direct targets of miR-210 [75]. Another study with A549 lung cells overexpressing miR-210 exhibited an aberrant mitochondrial phenotype, and additionally mRNA expression profiling analysis linked miR-210 to mitochondrial dysfunction [79]. Interestingly, as miR-210 is highly stable, when hypoxic cells undergo reoxygenation, HIF-1α is degraded immediately, but miR-210 remains stable to sustain glycolytic phenotype. This inhibits mitochondrial metabolism under normoxia and may contribute to Warburg effect in cancer cells. These evidences support a role of miR-210 in modulating mitochondrial metabolism, and thus facilitating adaptation of cancer cells to hypoxic condition.

As mentioned, the first process of catabolism of glucose is glycolysis, taking place out of mitochondria in cytoplasm. In context of miRNAs, it was found out that miR-155 indirectly upregulates hexokinase 2 (HK2), an enzyme involved in glucose phosphorylation, and, thus, may influence energy metabolism in breast cancer cells. Interestingly, one of two possible mechanisms mediating this miR-155-dependent

HK2 regulation includes miR-143 [66, 68, 80]. Other factor affecting glucose metabolism is alternative splicing of pyruvate kinase (PK), which splicing proteins are targeted by miR-124, miR-137, and miR-340. This miRNAs-dependent regulation of PK is able to impair colorectal cancer growth and counteract the Warburg effect [69, 81]. Moreover, PK is a direct target of the tumor-suppressive miR-326, which is thus also a potential regulator of a glucose metabolism [71, 73, 82].

In mitochondria, the regulation of energy metabolism can take place in both Krebs cycle and OXPHOS. Relatively to Krebs cycle, or TCA cycle, there are several miRNAs reported to be regulating several steps within the cycle, such as miR-183 downregulating isocitrate dehydrogenase or miR-743a downregulating malate dehydrogenase, all pushing the metabolism to a more glycolytic status [36, 83].

OXPHOS process take place in two major steps both coupled in inner mitochondrial membrane: the oxidation of nicotinamide adenine dinucleotide, its reduced form (NADH), or flavine adenine dinucleotide, its hydroquinone form ($FADH_2$), and the phosphorylation of ADP to form ATP [10, 83]. Presently there are also several studies reporting the regulation of OXPHOS by miRNAs, particularly: miR-210 downregulating Complex III in mitochondria; miR-181c, miR-210 and miR-338 downregulating Complex IV; and miR-141 downregulating Complex V in the mitochondrial ETC [36, 84].

In the last decade, miRNAs have emerged as critical regulators in cancer-related processes, being considered either oncogenic or tumor suppressive miRNAs (Fig. 8.2). Studies have shown that tumor-targeting therapies using miRNAs is becoming a novel diagnostic and therapeutic tool [85]. Particularly miR-200a has been demonstrated to suppress tumor growth in liver cancer [86], and also exhibited significantly lower expression in breast cancer [87], thus acting as a negative regulator or tumor suppressor for the cell growth, which is consistent with the role of miR-200a in hepatocellular carcinoma [88]. Several targets of miR-200a have been identified [89, 90]. Based on bioinformatics analysis, TFAM was predicted as one of miR-200a targets. TFAM is the primary transcription factor in mitochondria [91], being also implicated as a primary architectural protein of the mitochondrial genome by packing the mtDNA and a key regulator involved in mtDNA transcription and replication [92]. Furthermore, it was reported that TFAM expression is involved in tumor progression, cancer cell growth, and chemoresistance [93]. Accordingly, TFAM has been reported as a functional target of miR-200a in breast cancer cells. miR-200a overexpression significantly downregulated TFAM by directly targeting the 3′UTR of TFAM mRNA [87].

However, overexpression of TFAM without 3′UTR could overcome the inhibition by miR-200a and rescue the expression of TFAM, which could reverse the inhibition of TFAM-mediated mtDNA copy number by miR-200a [87]. Furthermore, miR-494 has been found to modulate mitochondrial biogenesis by downregulating the TFAM and the nuclear transcription factor Forkhead box J3 (FOXJ3) during myocyte differentiation and skeletal muscle adaptation to physical exercise [72].

A recent report suggests that p53-induced miR-34a plays an important role in nonsmall cell lung cancer (NSCLC) sensitization in response to capsaicin-induced DNA damage [94]. Bcl-2 was identified as the miR-34a target in these capsaicin-treated NSCLC cells. Downregulation of Bcl-2 created an anti-survival environment

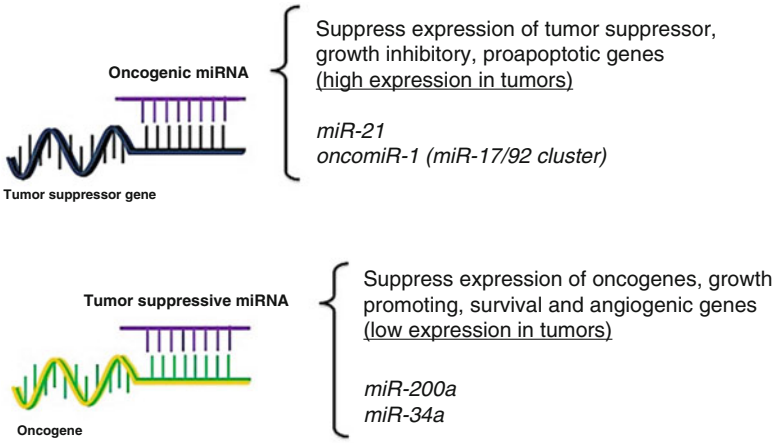

Fig. 8.2 microRNA activity in cancer: oncogenic or tumor suppressor role

in these NSCLC cells that favored Bax-mediated apoptosis via mitochondrial death cascade involving caspase-9 and -3, but not caspase-8, thereby ruling out the involvement of extrinsic death pathway. It is also accepted that p53-target miR-34a inhibits SIRT1 thereby deacetylating and stabilizing p53 in a positive feedback loop [95]. It is thus tempting to speculate that miR-34 may be an important player in modulating mitochondrial death pathway in cancer cells. Additionally, in some tumors the level of miR-21 is increased, and it suppresses the expression of PTEN, which regulates the mitophagy-associated PINK1 [70].

Concerning the role of miRNAs in cancer, one of the most studied pathways involves the miR-17/92 cluster, also known as oncomiR-1. There is increasing evidence on the oncogenic potential of this cluster of miRNAs [73, 96]. Being initially found amplified in diffuse cell lymphomas [80, 84], it was later shown, in B-cell lymphoma, that an ectopically overexpressed truncated version lacking miR-92 had oncogenic properties [81, 97]. Currently it has been described that this miRNA cluster can act as oncogene by suppressing apoptosis, and that oncomiR-1 is deregulated in B-cell lymphoma, B-cell chronic lymphocytic leukemia, acute myeloid leukemia, T-cell lymphoma, and particularly overexpressed in several types of cancer, such as osteosarcoma, neuroblastoma, neck, pancreatic, breast, lung, colorectal, ovarian, renal, and hepatic cancer [73, 82, 98].

miRNAs in Metabolic Disorders

Metabolic diseases are mainly characterized by the dysregulation, and consequent miscarriage of nutrient responsive genes or proteins to successfully coordinate pathways involved in the control of adaptive biological functions. Therefore, the

expression of genes involved in the regulation of metabolic function/signaling has to be finely tuned in order to properly respond to changes in metabolic status and then maintain cellular metabolic homeostasis. In addition to the classical transcriptional regulators, recent discoveries have shown the remarkable role of microRNAs in the posttranscriptional regulation of gene expression and their participation in many biological processes including regulation of metabolic function [99].

Being recently recognized as key regulators of metabolism, miRNAs have been found to act in one of the main organelles involved in metabolic pathways—mitochondria. Among the several roles that mitochondria play, energy metabolism is one of the most studied. As such, some miRNAs have already been identified as regulators in mitochondrial metabolism. miRNA-33 in particular has been shown to have a crucial role in cholesterol metabolism and also a conceivable use in clinical approaches [100].

Interestingly, some other miRNAs have been connected to lipid metabolism; miR-143 and -24 have been associated to increased incidence of metabolic syndrome through polymorphisms in miRNA binding sites in APOL6 3′UTR [101]. In addition, miR-24 and miR-126 have been associated to fatty acid metabolism [102], whereas miR-204-5p associates with the oxidation of fatty acids [103]. Trying to convey the notion that, since several miRNAs are predicted to target the same gene, and given that a single miRNA might modulate several genes, some authors describe the actions of miRNAs in metabolically relevant organs such as the pancreas, liver, and adipose tissue, and try to integrate the contribution of specific effects within the global landscape of lipid and carbohydrate homeostasis during metabolic syndrome and cardiometabolic disease [99].

Metabolic syndrome is a systemic disorder that includes a spectrum of abnormalities associated with obesity and type II diabetes. Defects in mitochondrial function, namely related to oxidation of fatty acids, have been linked to diet-induced obesity and the development of insulin resistance in adipose tissue and skeletal muscle [21, 104]. Consistently, obese individuals have mitochondria with compromised bioenergetic capacity [105, 106]. miRNAs that are involved in metabolism and metabolic disorders indicate an important regulatory role in the key organs involved in lipid and glucose metabolism [107]. Accordingly, certain serum circulating miRNA profiles have been shown to be dysregulated in obese children [108], putting forward the idea that some serum miRNAs could be used as a potential predictive tool for obesity and type 2 diabetes [109].

High-fat diet (HFD)plays a central role in the initiation of mitochondrial dysfunction that significantly contributes to skeletal muscle metabolic disorders in obesity. Given the emerging roles of miRNAs in the regulation of skeletal muscle metabolism, some authors started to study miRNAs involvement in mitochondrial function/dysfunction related to metabolic disorders. Certain studies addressed whether activation of a specific miRNA pathway would rescue the HFD-induced mitochondrial dysfunction via the sirtuin-1 (SIRT-1)/peroxisome proliferator-activated receptor γ coactivator-1a (PGC-1α) pathway [110].

Interestingly, miR-149 was shown to inhibit poly(ADP-ribose) polymerase-2 (PARP-2) and so increases cellular NAD+ levels and SIRT-1 activity that subsequently increases mitochondrial function and biogenesis via PGC-1α activation. Furthermore, skeletal muscles from HFD-fed obese mice exhibit low levels of miR-149 and high levels of PARP-2, and they show reduced mitochondrial function and biogenesis due to a decreased activation of the SIRT-1/PGC-1α pathway, suggesting that mitochondrial dysfunction in the skeletal muscle of obese mice may be because of, at least in part, miR-149 dysregulation [110].

Several miRNAs have been implicated in metabolic homeostasis based on loss-of-function studies in mice [107, 111]. miR-33, encoded by an intron of the sterol regulatory element binding protein (SREBP) gene, has been shown to collaborate with SREBP to regulate intracellular cholesterol levels and lipid homeostasis by targeting the adenosine triphosphate-binding cassette transporter A1, a regulator of cellular cholesterol efflux [112]. Other metabolic miRNAs, such as miR-103 and miR-107, regulate insulin and glucose homeostasis, whereas miRNAs such as miR-34a are emerging as key regulators of hepatic lipid homeostasis [107].

Some miRNAs have been linked to the regulation of glucose metabolism. Silencing of miR-103/107 improves glucose homeostasis and insulin sensitivity in mice [113]. Lin28a, which inhibits processing of let-7 miRNA, promotes insulin signaling and confers resistance to HFD-induced diabetes [114]. Conversely, let-7 overexpression impairs glucose tolerance and reduces insulin secretion in mice [115]. Additionally, it has been found that pharmacologic inhibition of miR-208a in mice confers resistance to obesity and improves insulin sensitivity [116].

Approximately one-third of miRNAs are encoded by introns of protein-coding genes, and frequently intronic miRNAs have been found to modulate, either positively or negatively, the same biological processes as the protein encoded by the host gene [117, 118]. Peroxisome proliferator-activated receptor γ coactivator 1β (PGC-1β)is a transcriptional coactivator that regulates metabolism and mitochondrial biogenesis through stimulation of nuclear hormone receptors and other transcription factors. The PGC-1β gene encodes two miRNAs, miR-378 and miR-378*, which counterbalance the metabolic actions of PGC-1β [119]. Mice genetically lacking miR-378 and miR-378* are resistant to HFD-induced obesity and exhibit enhanced mitochondrial fatty acid metabolism and elevated oxidative capacity of insulin-target tissues [119]. Among the many targets of these miRNAs, carnitine O-acetyltransferase, a mitochondrial enzyme involved in fatty acid metabolism, and MED13, a component of the Mediator complex that controls nuclear hormone receptor activity, are repressed by miR-378 and miR-378*, respectively, and are elevated in the livers of miR-378/378* KO mice.

The discovery of circulating miRNAs has highlighted their potential as both endocrine signaling molecules and disease markers. Deregulation of miRNAs may contribute to metabolic abnormalities, suggesting that miRNAs may potentially serve as therapeutic targets for ameliorating cardiometabolic disorders.

Another recent particular report has shown that HFD and excess fatty acids (FFAs) can cause enteric neuronal cell damage [120], contributing to delayed intestinal transit. The mechanism involve decreased enteric neuronal cell viability

through mitochondrial damage and ER stress, being miR-375 strongly implicated in mediating the detrimental effects of HFD (e.g., by downregulating the pro-survival protein Pdk1 translation) [120]. HFD rich in saturated FFA rises oxidative stress in many tissues, such as brain and gastric and intestinal mucosa [121]. Mitochondria, as the major source of ROS, are susceptible to FFA accumulation and the ROS-induced lipid peroxidation [122]. A recent study has showed a significant increase in mitochondrial SOD after palmitate exposure, suggesting a mitochondrial oxidative stress overload [120]. It was also reported a significant decrease in the mitochondrial quantity measured by cytochrome c oxidase IV protein level, as well as ultrastructural changes consistent with mitochondrial dysfunction, all of these effects being at least in part mediated by miR-375. Systemic injection of miR-375 inhibitor to HFD-fed mice prevented the development of detrimental effects of HFD on intestinal transit and enteric neurons, providing direct evidence for the role of miR-375 on modulating ER stress and mitochondrial function [120].

Since when miRNA emerged as a new class of epigenetic regulators of gene expression, there has been great interest in its role modulating metabolism and promoting the development and progression of metabolic disorders, namely diabetes-related chronic complications [123]. Abnormal levels of some miRNAs have been observed in the development of diabetic kidney disease [124]. Additionally, the development of diabetic nephropathy in rats has been linked to the regulation of NOX4 levels by miR-25, where NOX4 is a catalytic subunit of NADPH oxidase [125]. Furthermore, other studies have shown that, in *db*/*db* mice, the increased miR-21 levels halted the proliferation of mesangial cells and reduced the 24 h urine albumin releasing rate [126].

A fundamental function of mitochondria is to produce ATP via OXPHOS to supply energy for a variety of cellular functions. Unsurprisingly, also this vital function in mitochondria is targeted by miRNAs. miR-15b, miR-16, miR-195, and miR-424 have emerged as regulators of the ATP levels [9]. The overexpression of these miRNAs was shown to suppress ATP levels and affect mitochondrial integrity as well, by acting on their common target, the ADP-ribosylation factor-like 2 (ARL2) mRNA [127]. Additionally, upregulation of other miRNAs such as miR-15b or miR-195 has been reported to induce mitochondrial degeneration and ATP reduction in cardiomyocytes [9].

In mitochondria, the regulation of energy metabolism can take place in both Krebs cycle and OXPHOS. Relatively to Krebs cycle, or TCA cycle, there are several miRNAs reported to be regulating several steps within the cycle, such as miR-183 downregulating isocitrate dehydrogenase or miR-743a downregulating malate dehydrogenase, all pushing the metabolism to a more glycolytic status [36]. Currently, it is also known that some miRNAs modulate mitochondrial OXPHOS, through targeting several mitochondrial players involved in electron transfer and ATP production. In Table 1 we present a summary of miRNAs already reported to act in mitochondria and directly target some components involved in mitochondrial ETC. For example, miR-338 modulates OXPHOS and the mitochondrial function, targeting cytochrome c oxidase subunit IV (mt-COX4) mRNA in neurons [128].

OXPHOS process takes place in two major steps both coupled in inner mitochondrial membrane: the oxidation of nicotinamide adenine dinucleotide, its reduced form (NADH), or flavine adenine dinucleotide, its hydroquinone form (FADH2), and the phosphorylation of ADP to form ATP [10]. Presently there are also several studies reporting the regulation of OXPHOS by miRNAs, particularly: miR-210 downregulating Complex III in mitochondria; miR-181c, miR-210, and miR-338 downregulating Complex IV; and miR-141 downregulating Complex V in the mitochondrial ETC [36]. Particularly, some authors have already reported that the rat mitomiR miR-181c is involved in electron chain complex IV remodeling in cardiomyocytes [38].

Similarly to other reports on mitomiRs, rat cardiomyocyte mitochondria were found to harbor a unique miRNA expression pattern, with miR-181c enriched twofold in the mitochondrial fraction with respect to whole heart because of translocation of the miRNA into the organelles, for which cytochrome c oxidase subunit I (mt-COX1) mRNA was confirmed as a target for miR-181c [38]. The authors demonstrated that miR-181c, derived from the nuclear genome, translocates to the mitochondria, and more importantly, regulates mitochondrial gene expression and affects mitochondrial function. Coordination of nuclear gene expression and mitochondrial gene expression is thus essential. It was proven that in cardiomyocytes miRNA can regulate mitochondrial gene expression, specifically that miR-181c binds to the 3′-end of the mRNA of a mitochondrial gene, mt-COX1, a subunit of complex IV of the respiratory chain, and initially results in a decrease in mt-COX1 protein, complex IV remodeling, and increased production of ROS [38].

More recently, an in vivo method for administration of miR-181c in rats was used, displaying reduced exercise capacity and signs of heart failure, by targeting the 3′-end of mt-COX1 [129]. The mRNA levels of mitochondrial complex IV genes in the heart, but not any other mitochondrial genes, were significantly altered with miR-181c overexpression, suggesting selective mitochondrial complex IV remodeling due to miR-181c targeting mt-COX1. Isolated heart mitochondrial studies showed significantly altered O_2-consumption, ROS production, matrix calcium, and mitochondrial membrane potential in miR-181c-treated animals, showing that miRNA delivered to the heart in vivo can lead to cardiac dysfunction by regulating mitochondrial genes [129]. Moreover, other authors reported an important role for miR-181a in regulating the mitochondrial apoptotic pathway in cardiomyocytes challenged with oxidative stress [130]. The downregulation of miR-181a significantly inhibited H_2O_2-induced cellular apoptosis, ROS production, the increase in malondialdehyde (MDA) levels, the disruption of mitochondrial structure, and the activation of key signaling proteins in the mitochondrial apoptotic pathway. miR-181a/c may thus represent potential therapeutic targets for the treatment of cardiovascular diseases.

Regarding the involvement of mitochondria in energy metabolism and miRNA modulation is the newly described relationship between miR-26 and mitochondrial morphology in adipocytes. Adipose tissue contains thermogenic adipocytes (i.e., brown and brite/beige) that oxidize nutrients at exceptionally high rates via nonshivering thermogenesis. Its recent discovery in adult humans has opened up new

avenues to fight obesity and related disorders such as diabetes. miR-26 was found to be a key regulator of human white and brite adipocyte differentiation, being upregulated in early adipogenesis [131].

Intriguingly, miR-26a significantly induced pathways related to energy dissipation, shifted mitochondrial morphology toward that seen in brown adipocytes, and promoted uncoupled respiration by markedly increasing the hallmark protein of brown fat, uncoupling protein 1 (UCP1) [131]. miR-26a transfection evoked an increase in UCP1+ adipocytes up to 50 %. Ultrastructural analysis by transmission electron microscopy (TEM) further revealed a shift of mitochondrial morphology toward brown adipocyte characteristics as well as a slightly increased density of cristae. Additionally, mitochondria of adipocytes from miR-26a-transfected cells were bigger in size and more roundish (i.e., brown-like) in the late brite stage, indicating that miR-26a/b promote characteristics of energy-dissipating thermogenic adipocytes during human adipocyte differentiation.

Additionally, prohibitin (PHB)has also been reported to play a crucial role in adipocyte differentiation and mitochondrial function, a process also modulated by miRNA action [132]. The levels of both miR-27a and miR-27b were shown to be downregulated following adipogenic induction of human adipose-derived stem cells, whereas the mRNA level of PHB was upregulated. Moreover, overexpression of miR-27a or miR-27b inhibited PHB expression and adipocyte differentiation. More specifically, it was shown that ectopic expression of miR-27a or miR-27b impaired mitochondrial biogenesis, structure integrity, and Complex I activity accompanied by excessive ROS production [132], lightening an anti-adipogenic role of miR-27.

miRNAs in Neurodegeneration

The role of miRNAs has currently been extended to the neurodegenerative diseases' field. Altered expression of miRNAs is increasingly recognized as a feature of many disease states, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and Huntington's disease pathogenesis [83, 133, 134].

miRNAs are nowadays known for playing several important roles not only in neural differentiation during central nervous system (CNS) development but also in the progression of neurological disorders [135]. Some examples of the involvement of miRNAs in the nervous system have been reported, namely their regulatory roles in the expression of genes associated with spinal cord injury (SCI)-induced inflammation, oxidative stress, and apoptosis [136–138]. The findings have shown that miR-21 regulates astrocytic hypertrophy in injured spinal cords [139], and also alterations in the levels of miR-145 have been observed in neurons after sciatic nerve transection [140], as well as in injured spinal cords [136]. In rat hippocampal tissue, for example, it has been reported the presence of miRNA protein machinery in mitochondrial fractions [141].

Moreover, miRNAs were found in complexes co-immunoprecipitated from purified mitochondria, therefore displaying a role for mitochondria in regulating miRNA activity. More importantly, a subset of miRNAs was preferentially enriched in hippocampal mitochondria, but was found to be decreased following severe traumatic brain injury. Moreover, the levels of miR-155 and miR-223, both playing a role in inflammatory processes, were found to be elevated in cytoplasm and mitochondria, pointing out a role for mitochondrial regulation of miRNA expression in response to brain injury. Another study concerning neural damage found that miR-145 is mainly expressed in neurons and astrocytes located in the gray matter of the spinal cord. Using in vivo and in vitro genetic modification approaches, it has been demonstrated a novel role of miR-145 in the regulation of astrocytic dynamics, as well as its importance in astrocytes as a critical factor inducing astrogliosis after spinal cord injury [142].

Traditionally, conditions such as Parkinson's disease, Alzheimer's disease, and ALS have been considered as distinct entities; nevertheless, there is increasing evidence of clinical, pathological, and genetic overlap, culminating in a final common final pathway of neuronal cell death. The pathogenic mechanisms underlying neurodegeneration are complex, but the universal risk factor is aging, along with common features such as protein aggregation, neuroinflammation, and mitochondrial dysfunction. miRNAs are found in high abundance within the nervous system where they are key regulators of functions such as neurite outgrowth, dendritic spine morphology, neuronal differentiation, and synaptic plasticity [83, 143].

The onset of neurodegenerative disorders is very often associated with aging and senescence. Also in these biological phenomena miRNAs are playing a role, and tissue specific miRNA induction might have a protective function [144]. This might be the case for the aged brain-specific miR-22, which was demonstrated in a neurodegenerative model to exert a neuroprotective role by targeting factors implicated in Huntington's disease [145]. Similarly, miR-101a might have an Alzheimer's disease contrasting effect by inhibiting the production of Amyloid-beta precursor protein (APP) [146].

Alzheimer's Disease

Alzheimer's disease is a complex neurodegenerative disorder and the most common form of dementia in the elderly [84, 147]. The clinical signs of disease are a slow, progressive loss of cognitive function and memory loss, due to destruction of synapses and neurons, which ultimately leads to dementia and death. Alzheimer's disease is progressive with different brain regions and cells affected in a sequential process of increasing deposition of amyloid-β (Aβ) plaques and neurofibrillary tangles of hyperphosphorylated tau [96, 148].

The vast majority of Alzheimer's disease is sporadic, with no obvious genetic component, suggesting that other mechanisms are responsible. Recent studies have

demonstrated that alterations in the network of miRNAs contribute to the disease process, and several studies have used profiling strategies to show miRNA deregulation in Alzheimer's disease. Several miRNAs have been identified as deregulated including miR-107, miR-29, miR-9, miR-181, miR-34, miR-106, and miR-146 [83, 84]. Many of these have been linked to altered regulation of key genes known to be involved with Alzheimer's disease.

Downregulation of miR-107 at an early stage of Alzheimer's disease has been observed in temporal cortex and correlated with the upregulation of β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) in two studies, which could impact upon Aβ production [97, 149]. This finding was confirmed as being specific to miR-107 (and not a family member such as miR-103) and demonstrated that as miR-107 declines with advancing pathology, BACE1 increases along with neuritic plaque density [98, 150]. Interestingly, miR-107 and miR-124a, two miRNAs experimentally proven to target BACE1 also regulate other aspects of APP metabolism, thus demonstrating the capacity for single miRNAs to influence several components of the same pathway and the potential to produce additive effects. miR-107 directly targets a disintegrin and metalloproteinase 10 (ADAM10), another secretase enzyme which processes APP, and miR-124a is involved in the regulation of APP mRNA alternative splicing via direct targeting of polypyrimidine tract binding protein 1 (PTBP1) [133, 134, 151].

The miR-29 family of miRNAs has target sites on BACE1 mRNA and loss of this cluster is negatively correlated with BACE1 expression in a subset of sporadic Alzheimer's disease cases [143, 152]. In addition to regulating BACE1, miR-29a/b are increased in the aging brain and linked to modulation of microglial activation [147, 153]. The miR-29 cluster has been sequenced in a cohort of sporadic and familial patients and variants were found within the cluster that significantly associated with Alzheimer's disease [148, 154].

More recently, miR-339-5p has also been demonstrated to downregulate protein expression of BACE1 in human primary brain cultures and to be significantly reduced in brain tissue specimens of AD patients as compared with age-matched controls [155]. Therefore, miR-339-5p has been reported as a strong regulator of BACE1 expression in human brain cells and most likely dysregulated in at least a subset of AD patients, making this miRNA a novel drug target [155].

miR-9 is a highly conserved, brain enriched miRNA and the most frequently identified misregulated miRNA in Alzheimer's disease to date [83, 156]. Addition of Aβ to primary neuron cultures results in a rapid decrease of miR-9 in vitro and suggests that deregulation may be related to plaque formation [67, 149]. The targets for miR-9 include neurofilament heavy chain (NFH), a protein found in neurofibrillary tangles, and sirtuin 1 (SIRT1), a deacetylase that interacts with tau and is linked to accumulation of hyperphosphorylated forms of tau in the disease [150, 157]. Three other miRNAs have been found to suppress SIRT1, namely miR-181c, miR-34, and miR-132, all of which show consistent altered expression in Alzheimer's disease brain [151, 158].

Parkinson's Disease

Parkinson's disease (PD) is characterized clinically by bradykinesia, tremor, and rigidity. This is caused by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta. The majority of cases are idiopathic; however, around 20 % of patients have a positive family history. The most important and widely accepted monogenically inherited Parkinson's disease genes are α-synuclein (SNCA) and leucine-rich repeat kinase 2 (LRRK2) for late-onset disease and Parkin (PARK2), oncogene DJ1 (DJ1) and PTEN Induced Putative Kinase 1 (PINK1) for early onset [152, 159]. The neuropathology of Parkinson's disease is characterized by cellular inclusions known as Lewy bodies in neurons, the main components of which are α-synuclein, neurofilament, and ubiquitin.

Recent studies suggest that miRNAs may be involved in the development of Parkinson's disease. Of particular interest, because it not only relates miRNAs with neurodegeneration but also further relates miRNA with mitochondrial regulation and the onset of disease, is the case of complex-I targeting in neuronal cells [160]. The role of TNF-α in the regulation of cell death and miRNA-mediated mitochondrial functions was evaluated using neuronal cells SH-SY5Y (model of dopaminergic neuron degeneration similar to PD). It was found that increased levels of TNF-α alter the miRNAs targeting nuclear-encoded mitochondrial complex-I subunits, decreases ATP levels, increases ROS levels and mitochondrial turnover through autophagy, further inducing mitochondrial oxidative stress by dysregulation of complex-I activity and consequent cell death.

Deletion of Dicer (one of the regulators of miRNA expression machinery) in dopaminergic neurons in transgenic mice led to reduced locomotion and symptoms reminiscent of human Parkinson's disease [153, 161]. Expression profiling of miRNAs from patient midbrain samples revealed a significant decrease in miR-133b. miR-133b targets Pixt3, a transcription factor enriched in dopaminergic neurons, which is deficient in the aphakia mouse model of Parkinson's disease [154, 162]. A negative feedback model has been proposed to explain the relationship, in which, Pitx3 specifically induces transcription of miR-133b and Pitx3 activity is directly downregulated by miR-133b [83, 156].

miRNA profiling to evaluate deregulation of miRNAs in various regions of human Parkinson's disease brain tissue has also reported a widespread reduction in the miR-34b/c cluster, which could be detected early in the disease course. Depletion of these miRNAs in dopaminergic neuronal cells led to a reduction of cell viability accompanied by mitochondrial dysfunction [67, 163].

One of the most important factors in Parkinson's disease pathology is α-synuclein protein accumulation [157, 163]. Examination of the SNCA gene has revealed an unusually highly conserved and long 3′UTR sequence which is important in the posttranslational control of the gene and strongly suggests a role for miRNA regulation [158, 164]. Two miRNAs have been identified as directly targeting SNCA, namely miR-7 and miR-153. These brain-enriched miRNAs have been found to bind directly to SNCA mRNA and downregulate its expression [159, 165]. In addition, miR-7 suppresses SNCA mediated cytotoxicity in neuronal cell models [161, 166].

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is characterized by the progressive loss of upper and lower motor neurons from the motor cortex, brain stem and spinal cord. For the patient, this results in severe muscle atrophy leading to paralysis and death usually within 2–5 years of symptom onset [162, 167]. A family history of ALS is found in 5 % of patients, with the remaining 95 % of cases sporadic in nature. Several genes have now been identified as causative in ALS of which the most frequent are C9ORF72, superoxide dismutase 1 (SOD1), and transactive response DNA-binding protein (TARDBP) [83, 165].

Dicer knockdown was already used to generate transgenic mice lacking the ability to produce mature miRNAs in a subset of their postmitotic motor neurons [163, 168], in order to determine if miRNAs are essential to motor neuron survival. The transgenic animals showed progressive locomotor defects and denervation muscle atrophy caused by motor neuron loss. Further work revealed a specific increase in NFH expression, which was at least in part attributed to the loss of miR-9. This is a miRNA highly expressed in the brain and found to be upregulated in mouse models of the juvenile motor neuron disorder known as spinal muscular atrophy (SMA) [163, 169]. In addition, miRNAs that directly target neurofilament light chain (NFL) have been found to be altered in ALS. Upregulation of miR-146a and downregulation of miRNAs 524-5p and 582-3p were reported in SALS spinal cord [164, 170].

Changes in miRNAs have also been seen in peripheral ALS tissues, namely in the muscle from mutant SOD1 mouse models of ALS [165, 171]. A dramatic increase in the miR-206 was observed in transgenic mice at the time of symptom onset and was found to be a direct result of denervation. miR-206 is a skeletal muscle-enriched miRNA that has fundamental roles in muscle development and plasticity [166, 172]. A similar increase in miR-206 has also been observed in human ALS patient muscle tissue [167, 173]. The loss of miR-206 from transgenic SOD1 mice accelerated the rate of disease progression, most likely because miR-206 is a key player in nerve-muscle communication and therefore essential for reinnervation following nerve damage [165, 174].

Huntington's Disease

Huntington's disease is an autosomal dominant inherited disorder caused by an elongated CAG repeat expansion in the Huntingtin (HTT) gene. The classical motor symptom of chorea is not present in all patients, whilst other motor features such as impaired balance or abnormal fine finger movements are more likely to interfere with the patient's quality of life [168, 175]. Although HTT is ubiquitously expressed, the aggregates of mutant HTT protein, which are a pathological hallmark of the disease, are restricted to neuronal cells [169, 173, 176].

There are widespread gene expression changes in Huntington's disease and evidence suggests these can be attributed partly to miRNA deregulation [170, 175, 177]. Depletion of wild type HTT compromises miRNA mediated gene silencing and the mutant protein disrupts neuronal P body integrity [21, 104, 171]. There is also evidence to suggest other key components of miRNA biogenesis are deregulated in mouse models of the disease, including Dicer, Drosha, and Exportin-5, at different stages of the disease course [105, 106, 172]. Studies to profile miRNA expression in human tissue, mouse models of disease, and cellular systems have revealed numerous expression changes in miRNAs, suggesting that deregulation is extensive in Huntington's disease [107, 111, 173].

More specifically, the miR-200 family is altered in the cortex of mutant HTT mouse models at early stages of disease, which may compromise a network of genes involved in neuronal plasticity and survival [112, 174]. In cellular models of Huntington's disease, miR-146a, miR-125b, and miR-150 are downregulated while miR-34b was elevated by the presence of mutant HTT protein [175]. Further investigation revealed complex interplay between these miRNAs and several transcriptions factors, including p53, RelA, and NFkB, [173, 176]. Interestingly, miR-146a, miR-150, and miR-125b also targeted HTT and were predicted to interact with tata binding protein (TBP), a protein known to be recruited into mutant HTT aggregates, and were shown to modulate aggregate formation [175, 177].

In a recent study with a monkey model of Huntington, 11 miRNAs were found to be significantly associated with HD in the frontal cortex of the HD monkeys [178]. miR-128a, which has a corresponding disruption in humans and mice with HD, was downregulated in HD monkey model by the time of birth, and was also downregulated in the brains of pre-symptomatic and post-symptomatic HD patients. Additionally, the authors reported a panel of canonical HD signaling genes regulated by miR-128a as well, including HTT and Huntingtin Interaction Protein 1 (HIP1) [178].

While the clinical application of miRNAs as biomarkers and therapies is perhaps premature, the rate of discovery is promising. In an era of personalized medicine, the use of miRNA expression signatures to subclassify neurodegenerative disease, provide markers for therapeutic effectiveness and prognosis prediction, is an attractive prospect. Despite the anticipated off-target effects that cannot be fully predicted, saturation of the miRNA biogenesis pathway and possible immune activation, miRNA-based therapy has shown promise in animal models of neurodegeneration.

References

1. Tétreault N, De Guire V. miRNAs: their discovery, biogenesis and mechanism of action. Clin Biochem. 2013;46(10–11):842–5.
2. Ambros V. The functions of animal microRNAs. Nature. 2004;431(7006):350–5.
3. D'Aquila P, Bellizzi D, Passarino G. Mitochondria in health, aging and diseases: the epigenetic perspective. Biogerontology. 2015;25.

4. Hudson G, Gomez-Duran A, Wilson IJ, Chinnery PF. Recent mitochondrial DNA mutations increase the risk of developing common late-onset human diseases. PLoS Genet. 2014;10(5), e1004369.
5. Dowling DK. Evolutionary perspectives on the links between mitochondrial genotype and disease phenotype. Biochim Biophys Acta. 2014;1840(4):1393–403.
6. Guha M, Avadhani NG. Mitochondrial retrograde signaling at the crossroads of tumor bioenergetics, genetics and epigenetics. Mitochondrion. 2013;13(6):577–91.
7. Whelan SP, Zuckerbraun BS. Mitochondrial signaling: forwards, backwards, and in between. Oxid Med Cell Longev. 2013;2013:351613.
8. Duarte FV, Palmeira CM, Rolo AP. The role of microRNAs in mitochondria: small players acting wide. Genes (Basel). 2014;5(4):865–86.
9. Tomasetti M, Neuzil J, Dong L. MicroRNAs as regulators of mitochondrial function: role in cancer suppression. Biochim Biophys Acta. 2014;1840(4):1441–53.
10. Nunnari J, Suomalainen A. Mitochondria: in sickness and in health. Cell. 2012;148(6): 1145–59.
11. Kushnareva Y, Newmeyer DD. Bioenergetics and cell death. Ann N Y Acad Sci. 2010;1201:50–7.
12. Michel S, Wanet A, De Pauw A, Rommelaere G, Arnould T, Renard P. Crosstalk between mitochondrial (dys)function and mitochondrial abundance. J Cell Physiol. 2012;227(6): 2297–310.
13. DiMauro S. A history of mitochondrial diseases. J Inherit Metab Dis. 2011;34(2):261–76.
14. Zorzano A, Hernández-Alvarez MI, Palacín M, Mingrone G. Alterations in the mitochondrial regulatory pathways constituted by the nuclear co-factors PGC-1alpha or PGC-1beta and mitofusin 2 in skeletal muscle in type 2 diabetes. Biochim Biophys Acta. 2010; 1797(6–7):1028–33.
15. Peralta S, Wang X, Moraes CT. Mitochondrial transcription: lessons from mouse models. Biochim Biophys Acta. 2012;1819(9–10):961–9.
16. Krishnan KJ, Greaves LC, Reeve AK, Turnbull D. The ageing mitochondrial genome. Nucleic Acids Res. 2007;35(22):7399–405.
17. Larsson N-G. Somatic mitochondrial DNA mutations in mammalian aging. Annu Rev Biochem. 2010;79:683–706.
18. Wu Y-T, Wu S-B, Lee W-Y, Wei Y-H. Mitochondrial respiratory dysfunction-elicited oxidative stress and posttranslational protein modification in mitochondrial diseases. Ann N Y Acad Sci. 2010;1201:147–56.
19. Rolo AP, Palmeira CM. Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. Toxicol Appl Pharmacol. 2006;212(2):167–78.
20. Palmeira CM, Rolo AP, Berthiaume J, Bjork JA, Wallace KB. Hyperglycemia decreases mitochondrial function: the regulatory role of mitochondrial biogenesis. Toxicol Appl Pharmacol. 2007;225(2):214–20.
21. Rolo AP, Gomes AP, Palmeira CM. Regulation of mitochondrial biogenesis in metabolic syndrome. Curr Drug Targets. 2011;12(6):872–8.
22. Gomes AP, Duarte FV, Nunes P, Hubbard BP, Teodoro JS, Varela AT, et al. Berberine protects against high fat diet-induced dysfunction in muscle mitochondria by inducing SIRT1-dependent mitochondrial biogenesis. Biochim Biophys Acta. 2012;1822(2):185–95.
23. Perier C, Vila M. Mitochondrial biology and Parkinson's disease. Cold Spring Harb Perspect Med. 2012;2(2):a009332.
24. Chen H, Chan DC. Physiological functions of mitochondrial fusion. Ann N Y Acad Sci. 2010;1201:21–5.
25. Twig G, Elorza A, Molina AJA, Mohamed H, Wikstrom JD, Walzer G, et al. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J. 2008;27(2):433–46.
26. Westermann B. Mitochondrial fusion and fission in cell life and death. Nat Rev Mol Cell Biol. 2010;11(12):872–84.

27. Amodio N, Leotta M, Bellizzi D, Di Martino MT, D'Aquila P, Lionetti M, et al. DNA-demethylating and anti-tumor activity of synthetic miR-29b mimics in multiple myeloma. Oncotarget. 2012;3(10):1246–58.
28. Duchen MR. Mitochondria in health and disease: perspectives on a new mitochondrial biology. Mol Aspects Med. 2004;25(4):365–451.
29. Kren BT, Wong PY-P, Sarver A, Zhang X, Zeng Y, Steer CJ. MicroRNAs identified in highly purified liver-derived mitochondria may play a role in apoptosis. RNA Biol. 2009;6(1): 65–72.
30. Rackham O, Shearwood A-MJ, Mercer TR, Davies SMK, Mattick JS, Filipovska A. Long noncoding RNAs are generated from the mitochondrial genome and regulated by nuclear-encoded proteins. RNA. 2011;17(12):2085–93.
31. Yang K-C, Yamada KA, Patel AY, Topkara VK, George I, Cheema FH, et al. Deep RNA sequencing reveals dynamic regulation of myocardial noncoding RNAs in failing human heart and remodeling with mechanical circulatory support. Circulation. 2014;129(9): 1009–21.
32. Lung B, Zemann A, Madej MJ, Schuelke M, Techritz S, Ruf S, et al. Identification of small non-coding RNAs from mitochondria and chloroplasts. Nucleic Acids Res. 2006;34(14):3842–52.
33. Ro S, Ma H-Y, Park C, Ortogero N, Song R, Hennig GW, et al. The mitochondrial genome encodes abundant small noncoding RNAs. Cell Res. 2013;23(6):759–74.
34. Bandiera S, Matégot R, Girard M, Demongeot J, Henrion-Caude A. MitomiRs delineating the intracellular localization of microRNAs at mitochondria. Free Radic Biol Med. 2013;64:12–9.
35. Sripada L, Tomar D, Singh R. Mitochondria: one of the destinations of miRNAs. Mitochondrion. 2012;12(6):593–9.
36. Bienertova-Vasku J, Sana J, Slaby O. The role of microRNAs in mitochondria in cancer. Cancer Lett. 2013;336(1):1–7.
37. Shinde S, Bhadra U. A complex genome-microRNA interplay in human mitochondria. Biomed Res Int. 2015;2015:206382.
38. Das S, Ferlito M, Kent OA, Fox-Talbot K, Wang R, Liu D, et al. Nuclear miRNA regulates the mitochondrial genome in the heart. Circ Res. 2012;110(12):1596–603.
39. Li P, Jiao J, Gao G, Prabhakar BS. Control of mitochondrial activity by miRNAs. J Cell Biochem. 2012;113(4):1104–10.
40. Latronico MVG, Condorelli G. The might of microRNA in mitochondria. Circ Res. 2012;110(12):1540–2.
41. Bandiera S, Rüberg S, Girard M, Cagnard N, Hanein S, Chrétien D, et al. Nuclear outsourcing of RNA interference components to human mitochondria. PLoS One. 2011;6(6), e20746.
42. Barrey E, Saint-Auret G, Bonnamy B, Damas D, Boyer O, Gidrol X. Pre-microRNA and mature microRNA in human mitochondria. PLoS One. 2011;6(5), e20220.
43. Zhang X, Zuo X, Yang B, Li Z, Xue Y, Zhou Y, et al. MicroRNA directly enhances mitochondrial translation during muscle differentiation. Cell. 2014;158(3):607–19.
44. Chan DC. Fusion and fission: interlinked processes critical for mitochondrial health. Annu Rev Genet. 2012;46:265–87.
45. Shutt TE, McBride HM. Staying cool in difficult times: mitochondrial dynamics, quality control and the stress response. Biochim Biophys Acta. 2013;1833(2):417–24.
46. Wang J-X, Jiao J-Q, Li Q, Long B, Wang K, Liu J-P, et al. miR-499 regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1. Nat Med. 2011;17(1): 71–8.
47. Li J, Donath S, Li Y, Qin D, Prabhakar BS, Li P. miR-30 regulates mitochondrial fission through targeting p53 and the dynamin-related protein-1 pathway. PLoS Genet. 2010;6(1), e1000795.
48. Li X, Wang FS, Wu ZY, Lin JL, Lan WB, Lin JH. MicroRNA-19b targets Mfn1 to inhibit Mfn1-induced apoptosis in osteosarcoma cells. Neoplasma. 2014;61(3):265–73.

49. Zhang Y, Yang L, Gao Y-F, Fan Z-M, Cai X-Y, Liu M-Y, et al. MicroRNA-106b induces mitochondrial dysfunction and insulin resistance in C2C12 myotubes by targeting mitofusin-2. Mol Cell Endocrinol. 2013;381(1–2):230–40.
50. Tak H, Kim J, Jayabalan AK, Lee H, Kang H, Cho D-H, et al. miR-27 regulates mitochondrial networks by directly targeting the mitochondrial fission factor. Exp Mol Med. 2014;46, e123.
51. Long B, Wang K, Li N, Murtaza I, Xiao J-Y, Fan Y-Y, et al. miR-761 regulates the mitochondrial network by targeting mitochondrial fission factor. Free Radic Biol Med. 2013;65C: 371–9.
52. Li J, Li Y, Jiao J, Wang J, Li Y, Qin D, et al. Mitofusin 1 is negatively regulated by microRNA 140 in cardiomyocyte apoptosis. Mol Cell Biol. 2014;34(10):1788–99.
53. Wang K, Long B, Jiao J-Q, Wang J-X, Liu J-P, Li Q, et al. miR-484 regulates mitochondrial network through targeting Fis1. Nat Commun. 2012;3:781.
54. Yoon Y, Galloway CA, Jhun BS, Yu T. Mitochondrial dynamics in diabetes. Antioxid Redox Signal. 2011;14(3):439–57.
55. Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, et al. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. J Biol Chem. 2008;283(16):10892–903.
56. Kundu M, Lindsten T, Yang C-Y, Wu J, Zhao F, Zhang J, et al. Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. Blood. 2008;112(4):1493–502.
57. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57–70.
58. Frankel LB, Wen J, Lees M, Høyer-Hansen M, Farkas T, Krogh A, et al. microRNA-101 is a potent inhibitor of autophagy. EMBO J. 2011;30(22):4628–41.
59. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5): 646–74.
60. Xiao J, Zhu X, Bin H, Zhang Y, Kang B, Wang Z, et al. MiR-204 regulates cardiomyocyte autophagy induced by ischemia-reperfusion through LC3-II. J Biomed Sci. 2011;18(1):35.
61. Warburg O. On respiratory impairment in cancer cells. Science. 1956;124(3215):269–70.
62. Zhu H, Wu H, Liu X, Li B, Chen Y, Ren X, et al. Regulation of autophagy by a beclin 1-targeted microRNA, miR-30a, in cancer cells. Autophagy. 2009;5(6):816–23.
63. Zheng J. Energy metabolism of cancer: glycolysis versus oxidative phosphorylation (review). Oncol Lett. 2012;4(6):1151–7.
64. Fogg VC, Lanning NJ, Mackeigan JP. Mitochondria in cancer: at the crossroads of life and death. Chin J Cancer. 2011;30(8):526–39.
65. Frankel LB, Lund AH. MicroRNA regulation of autophagy. Carcinogenesis. 2012;33(11): 2018–25.
66. Fang R, Xiao T, Fang Z, Sun Y, Li F, Gao Y, et al. MicroRNA-143 (miR-143) regulates cancer glycolysis via targeting hexokinase 2 gene. J Biol Chem. 2012;287(27):23227–35.
67. Minones-Moyano E, Porta S, Escaramis G, Rabionet R, Iraola S, Kagerbauer B, et al. MicroRNA profiling of Parkinson's disease brains identifies early downregulation of miR-34b/c which modulate mitochondrial function. Hum Mol Genet. 2011;20(15):3067–78.
68. Jiang S, Zhang L-F, Zhang H-W, Hu S, Lu M-H, Liang S, et al. A novel miR-155/miR-143 cascade controls glycolysis by regulating hexokinase 2 in breast cancer cells. EMBO J. 2012;31(8):1985–98.
69. Sun Y, Zhao X, Zhou Y, Hu Y. miR-124, miR-137 and miR-340 regulate colorectal cancer growth via inhibition of the Warburg effect. Oncol Rep. 2012;28(4):1346–52.
70. Zhang J-G, Wang J-J, Zhao F, Liu Q, Jiang K, Yang G-H. MicroRNA-21 (miR-21) represses tumor suppressor PTEN and promotes growth and invasion in non-small cell lung cancer (NSCLC). Clin Chim Acta. 2010;411(11–12):846–52.
71. Kefas B, Comeau L, Erdle N, Montgomery E, Amos S, Purow B. Pyruvate kinase M2 is a target of the tumor-suppressive microRNA-326 and regulates the survival of glioma cells. Neuro Oncol. 2010;12(11):1102–12.

72. Yamamoto H, Morino K, Nishio Y, Ugi S, Yoshizaki T, Kashiwagi A, et al. MicroRNA-494 regulates mitochondrial biogenesis in skeletal muscle through mitochondrial transcription factor A and Forkhead box j3. Am J Physiol Endocrinol Metab. 2012;303(12):E1419–27.
73. Mogilyansky E, Rigoutsos I. The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. Cell Death Differ. 2013;20(12):1603–14.
74. Gee HE, Ivan C, Calin GA, Ivan M. HypoxamiRs and cancer: from biology to targeted therapy. Antioxid Redox Signal. 2014;21(8):1220–38.
75. Qin Q, Furong W, Baosheng L. Multiple functions of hypoxia-regulated miR-210 in cancer. J Exp Clin Cancer Res. 2014;33:50.
76. Camps C, Buffa FM, Colella S, Moore J, Sotiriou C, Sheldon H, et al. hsa-miR-210 Is induced by hypoxia and is an independent prognostic factor in breast cancer. Clin Cancer Res. 2008;14(5):1340–8.
77. Gee HE, Camps C, Buffa FM, Patiar S, Winter SC, Betts G, et al. hsa-mir-210 is a marker of tumor hypoxia and a prognostic factor in head and neck cancer. Cancer. 2010;116(9): 2148–58.
78. Chan SY, Zhang Y-Y, Hemann C, Mahoney CE, Zweier JL, Loscalzo J. MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2. Cell Metab. 2009;10(4):273–84.
79. Puisségur M-P, Mazure NM, Bertero T, Pradelli L, Grosso S, Robbe-Sermesant K, et al. miR-210 is overexpressed in late stages of lung cancer and mediates mitochondrial alterations associated with modulation of HIF-1 activity. Cell Death Differ. 2011;18(3):465–78.
80. Ota A, Tagawa H, Karnan S, Tsuzuki S, Karpas A, Kira S, et al. Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. Cancer Res. 2004;64(9):3087–95.
81. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, et al. A microRNA polycistron as a potential human oncogene. Nature. 2005;435(7043):828–33.
82. Olive V, Jiang I, He L. mir-17-92, a cluster of miRNAs in the midst of the cancer network. Int J Biochem Cell Biol. 2010;42(8):1348–54.
83. Goodall EF, Heath PR, Bandmann O, Kirby J, Shaw PJ. Neuronal dark matter: the emerging role of microRNAs in neurodegeneration. Front Cell Neurosci. 2013;7:178.
84. Schonrock N, Götz J. Decoding the non-coding RNAs in Alzheimer's disease. Cell Mol Life Sci. 2012;69(21):3543–59.
85. Krell J, Frampton AE, Stebbing J. MicroRNAs in the cancer clinic. Front Biosci (Elite Ed). 2013;5:204–13.
86. Radojicic J, Zaravinos A, Vrekoussis T, Kafousi M, Spandidos DA, Stathopoulos EN. MicroRNA expression analysis in triple-negative (ER, PR and Her2/neu) breast cancer. Cell Cycle. 2011;10(3):507–17.
87. Yao J, Zhou E, Wang Y, Xu F, Zhang D, Zhong D. microRNA-200a inhibits cell proliferation by targeting mitochondrial transcription factor A in breast cancer. DNA Cell Biol. 2014;33(5):291–300.
88. Zhang L, Yang F, Yuan J-H, Yuan S-X, Zhou W-P, Huo X-S, et al. Epigenetic activation of the MiR-200 family contributes to H19-mediated metastasis suppression in hepatocellular carcinoma. Carcinogenesis. 2013;34(3):577–86.
89. Roybal JD, Zang Y, Ahn Y-H, Yang Y, Gibbons DL, Baird BN, et al. miR-200 Inhibits lung adenocarcinoma cell invasion and metastasis by targeting Flt1/VEGFR1. Mol Cancer Res. 2011;9(1):25–35.
90. Rasheed SAK, Teo CR, Beillard EJ, Voorhoeve PM, Casey PJ. MicroRNA-182 and microRNA-200a control G-protein subunit α-13 (GNA13) expression and cell invasion synergistically in prostate cancer cells. J Biol Chem. 2013;288(11):7986–95.
91. Bonawitz ND, Clayton DA, Shadel GS. Initiation and beyond: multiple functions of the human mitochondrial transcription machinery. Mol Cell. 2006;24(6):813–25.

92. Lee H-C, Wei Y-H. Mitochondrial DNA instability and metabolic shift in human cancers. Int J Mol Sci. 2009;10(2):674–701.
93. Han B, Izumi H, Yasuniwa Y, Akiyama M, Yamaguchi T, Fujimoto N, et al. Human mitochondrial transcription factor A functions in both nuclei and mitochondria and regulates cancer cell growth. Biochem Biophys Res Commun. 2011;408(1):45–51.
94. Chakraborty S, Mazumdar M, Mukherjee S, Bhattacharjee P, Adhikary A, Manna A, et al. Restoration of p53/miR-34a regulatory axis decreases survival advantage and ensures Bax-dependent apoptosis of non-small cell lung carcinoma cells. FEBS Lett. 2014;588(4): 549–59.
95. Yamakuchi M, Lowenstein CJ. MiR-34, SIRT1 and p53: the feedback loop. Cell Cycle. 2009;8(5):712–5.
96. Braak H, Braak E. Staging of Alzheimer's disease-related neurofibrillary changes. Neurobiol Aging. 1995;16(3):271–8; discussion 278–84.
97. Nelson PT, Wang W-X. MiR-107 is reduced in Alzheimer's disease brain neocortex: validation study. J Alzheimers Dis. 2010;21(1):75–9.
98. Wang W-X, Rajeev BW, Stromberg AJ, Ren N, Tang G, Huang Q, et al. The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1. J Neurosci. 2008;28(5):1213–23.
99. Price NL, Ramírez CM, Fernández-Hernando C. Relevance of microRNA in metabolic diseases. Crit Rev Clin Lab Sci. 2014;51(6):305–20.
100. Rayner KJ, Esau CC, Hussain FN, McDaniel AL, Marshall SM, van Gils JM, et al. Inhibition of miR-33a/b in non-human primates raises plasma HDL and lowers VLDL triglycerides. Nature. 2011;478(7369):404–7.
101. Ye Q, Zhao X, Xu K, Li Q, Cheng J, Gao Y, et al. Polymorphisms in lipid metabolism related miRNA binding sites and risk of metabolic syndrome. Gene. 2013;528(2):132–8.
102. Liang T, Liu C, Ye Z. Deep sequencing of small RNA repertoires in mice reveals metabolic disorders-associated hepatic miRNAs. PLoS One. 2013;8(11), e80774.
103. Civelek M, Hagopian R, Pan C, Che N, Yang W-P, Kayne PS, et al. Genetic regulation of human adipose microRNA expression and its consequences for metabolic traits. Hum Mol Genet. 2013;22(15):3023–37.
104. Maassen JA, Romijn JA, Heine RJ. Fatty acid-induced mitochondrial uncoupling in adipocytes as a key protective factor against insulin resistance and beta cell dysfunction: a new concept in the pathogenesis of obesity-associated type 2 diabetes mellitus. Diabetologia. 2007;50(10):2036–41.
105. Anderson EJ, Lustig ME, Boyle KE, Woodlief TL, Kane DA, Lin C-T, et al. Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. J Clin Invest. 2009;119(3):573–81.
106. Li J, Romestaing C, Han X, Li Y, Hao X, Wu Y, et al. Cardiolipin remodeling by ALCAT1 links oxidative stress and mitochondrial dysfunction to obesity. Cell Metab. 2010;12(2):154–65.
107. Rottiers V, Näär AM. MicroRNAs in metabolism and metabolic disorders. Nat Rev Mol Cell Biol. 2012;13(4):239–50.
108. Koch L. Paediatrics: circulating microRNAs-predictors of obesity? Nat Rev Endocrinol. 2013;9(10):565.
109. Pescador N, Pérez-Barba M, Ibarra JM, Corbatón A, Martínez-Larrad MT, Serrano-Ríos M. Serum circulating microRNA profiling for identification of potential type 2 diabetes and obesity biomarkers. PLoS One. 2013;8(10), e77251.
110. Mohamed JS, Hajira A, Pardo PS, Boriek AM. MicroRNA-149 inhibits PARP-2 and promotes mitochondrial biogenesis via SIRT-1/PGC-1α network in skeletal muscle. Diabetes. 2014;63(5):1546–59.
111. Näär AM. MiRs with a sweet tooth. Cell Metab. 2011;14(2):149–50.

112. Najafi-Shoushtari SH, Kristo F, Li Y, Shioda T, Cohen DE, Gerszten RE, et al. MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. Science. 2010;328(5985):1566–9.
113. Trajkovski M, Hausser J, Soutschek J, Bhat B, Akin A, Zavolan M, et al. MicroRNAs 103 and 107 regulate insulin sensitivity. Nature. 2011;474(7353):649–53.
114. Zhu H, Shyh-Chang N, Segrè AV, Shinoda G, Shah SP, Einhorn WS, et al. The Lin28/let-7 axis regulates glucose metabolism. Cell. 2011;147(1):81–94.
115. Frost RJA, Olson EN. Control of glucose homeostasis and insulin sensitivity by the Let-7 family of microRNAs. Proc Natl Acad Sci U S A. 2011;108(52):21075–80.
116. Grueter CE, van Rooij E, Johnson BA, DeLeon SM, Sutherland LB, Qi X, et al. A cardiac microRNA governs systemic energy homeostasis by regulation of MED13. Cell. 2012;149(3):671–83.
117. van Rooij E, Quiat D, Johnson BA, Sutherland LB, Qi X, Richardson JA, et al. A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. Dev Cell. 2009;17(5):662–73.
118. Small EM, Olson EN. Pervasive roles of microRNAs in cardiovascular biology. Nature. 2011;469(7330):336–42.
119. Carrer M, Liu N, Grueter CE, Williams AH, Frisard MI, Hulver MW, et al. Control of mitochondrial metabolism and systemic energy homeostasis by microRNAs 378 and 378. Proc Natl Acad Sci U S A. 2012;109(38):15330–5.
120. Nezami BG, Mwangi SM, Lee JE, Jeppsson S, Anitha M, Yarandi SS, et al. MicroRNA 375 mediates palmitate-induced enteric neuronal damage and high-fat diet-induced delayed intestinal transit in mice. Gastroenterology. 2014;146(2):473–83.e3.
121. Stranahan AM, Cutler RG, Button C, Telljohann R, Mattson MP. Diet-induced elevations in serum cholesterol are associated with alterations in hippocampal lipid metabolism and increased oxidative stress. J Neurochem. 2011;118(4):611–5.
122. Schrauwen P, Schrauwen-Hinderling V, Hoeks J, Hesselink MKC. Mitochondrial dysfunction and lipotoxicity. Biochim Biophys Acta. 2010;1801(3):266–71.
123. Wegner M, Neddermann D, Piorunska-Stolzmann M, Jagodzinski PP. Role of epigenetic mechanisms in the development of chronic complications of diabetes. Diabetes Res Clin Pract. 2014;105(2):164–75.
124. Alvarez ML, Distefano JK. The role of non-coding RNAs in diabetic nephropathy: potential applications as biomarkers for disease development and progression. Diabetes Res Clin Pract. 2013;99(1):1–11.
125. Fu Y, Zhang Y, Wang Z, Wang L, Wei X, Zhang B, et al. Regulation of NADPH oxidase activity is associated with miRNA-25-mediated NOX4 expression in experimental diabetic nephropathy. Am J Nephrol. 2010;32(6):581–9.
126. Zhang Z, Peng H, Chen J, Chen X, Han F, Xu X, et al. MicroRNA-21 protects from mesangial cell proliferation induced by diabetic nephropathy in db/db mice. FEBS Lett. 2009;583(12):2009–14.
127. Nishi H, Ono K, Iwanaga Y, Horie T, Nagao K, Takemura G, et al. MicroRNA-15b modulates cellular ATP levels and degenerates mitochondria via Arl2 in neonatal rat cardiac myocytes. J Biol Chem. 2010;285(7):4920–30.
128. Aschrafi A, Schwechter AD, Mameza MG, Natera-Naranjo O, Gioio AE, Kaplan BB. MicroRNA-338 regulates local cytochrome c oxidase IV mRNA levels and oxidative phosphorylation in the axons of sympathetic neurons. J Neurosci. 2008;28(47):12581–90.
129. Das S, Bedja D, Campbell N, Dunkerly B, Chenna V, Maitra A, et al. miR-181c regulates the mitochondrial genome, bioenergetics, and propensity for heart failure in vivo. PLoS One. 2014;9(5), e96820.
130. Wang L, Huang H, Fan Y, Kong B, Hu H, Hu K, et al. Effects of downregulation of microRNA-181a on H2O2-induced H9c2 cell apoptosis via the mitochondrial apoptotic pathway. Oxid Med Cell Longev. 2014;2014:960362.

131. Karbiener M, Pisani DF, Frontini A, Oberreiter LM, Lang E, Vegiopoulos A, et al. MicroRNA-26 family is required for human adipogenesis and drives characteristics of brown adipocytes. Stem Cells. 2014;32(6):1578–90.
132. Kang T, Lu W, Xu W, Anderson L, Bacanamwo M, Thompson W, et al. MicroRNA-27 (miR-27) targets prohibitin and impairs adipocyte differentiation and mitochondrial function in human adipose-derived stem cells. J Biol Chem. 2013;288(48):34394–402.
133. Smith P, Hashimi Al A, Girard J, Delay C, Hébert SS. In vivo regulation of amyloid precursor protein neuronal splicing by microRNAs. J Neurochem. 2011;116(2):240–7.
134. Augustin R, Endres K, Reinhardt S, Kuhn P-H, Lichtenthaler SF, Hansen J, et al. Computational identification and experimental validation of microRNAs binding to the Alzheimer-related gene ADAM10. BMC Med Genet. 2012;13:35.
135. Bian S, Xu T-L, Sun T. Tuning the cell fate of neurons and glia by microRNAs. Curr Opin Neurobiol. 2013;23(6):928–34.
136. Liu N-K, Wang X-F, Lu Q-B, Xu X-M. Altered microRNA expression following traumatic spinal cord injury. Exp Neurol. 2009;219(2):424–9.
137. Strickland ER, Hook MA, Balaraman S, Huie JR, Grau JW, Miranda RC. MicroRNA dysregulation following spinal cord contusion: implications for neural plasticity and repair. Neuroscience. 2011;186:146–60.
138. Yunta M, Nieto-Díaz M, Esteban FJ, Caballero-López M, Navarro-Ruíz R, Reigada D, et al. MicroRNA dysregulation in the spinal cord following traumatic injury. PLoS One. 2012;7(4), e34534.
139. Bhalala OG, Pan L, Sahni V, McGuire TL, Gruner K, Tourtellotte WG, et al. microRNA-21 regulates astrocytic response following spinal cord injury. J Neurosci. 2012;32(50): 17935–47.
140. Zhang H-Y, Zheng S-J, Zhao J-H, Zhao W, Zheng L-F, Zhao D, et al. MicroRNAs 144, 145, and 214 are down-regulated in primary neurons responding to sciatic nerve transection. Brain Res. 2011;1383:62–70.
141. Wang W-X, Visavadiya NP, Pandya JD, Nelson PT, Sullivan PG, Springer JE. Mitochondria-associated microRNAs in rat hippocampus following traumatic brain injury. Exp Neurol. 2015;265:84–93.
142. Wang C-Y, Yang S-H, Tzeng S-F. MicroRNA-145 as one negative regulator of astrogliosis. Glia. 2014;63(2):194–205.
143. Zong Y, Wang H, Dong W, Quan X, Zhu H, Xu Y, et al. miR-29c regulates BACE1 protein expression. Brain Res. 2011;1395:108–15.
144. Lauri A, Pompilio G, Capogrossi MC. The mitochondrial genome in aging and senescence. Ageing Res Rev. 2014;18C:1–15.
145. Jovicic A, Zaldivar Jolissaint JF, Moser R, Mde Silva Santos F, Luthi-Carter R. MicroRNA-22 (miR-22) overexpression is neuroprotective via general anti-apoptotic effects and may also target specific Huntington's disease-related mechanisms. PLoS One. 2013;8(1), e54222.
146. Long JM, Lahiri DK. MicroRNA-101 downregulates Alzheimer's amyloid-β precursor protein levels in human cell cultures and is differentially expressed. Biochem Biophys Res Commun. 2011;404(4):889–95.
147. Fenn AM, Smith KM, Lovett-Racke AE, Guerau-de-Arellano M, Whitacre CC, Godbout JP. Increased micro-RNA 29b in the aged brain correlates with the reduction of insulin-like growth factor-1 and fractalkine ligand. Neurobiol Aging. 2013;34(12):2748–58.
148. Bettens K, Brouwers N, Engelborghs S, Van Miegroet H, De Deyn PP, Theuns J, et al. APP and BACE1 miRNA genetic variability has no major role in risk for Alzheimer disease. Hum Mutat. 2009;30(8):1207–13.
149. Schonrock N, Ke YD, Humphreys D, Staufenbiel M, Ittner LM, Preiss T, et al. Neuronal microRNA deregulation in response to Alzheimer's disease amyloid-beta. PLoS One. 2010;5(6), e11070.
150. Liu Q, Xie F, Alvarado-Diaz A, Smith MA, Moreira PI, Zhu X, et al. Neurofilamentopathy in neurodegenerative diseases. Open Neurol J. 2011;5:58–62.

151. Wong H-KA, Veremeyko T, Patel N, Lemere CA, Walsh DM, Esau C, et al. De-repression of FOXO3a death axis by microRNA-132 and -212 causes neuronal apoptosis in Alzheimer's disease. Hum Mol Genet. 2013;22(15):3077–92.
152. Coppedè F. Genetics and epigenetics of Parkinson's disease. ScientificWorldJournal. 2012;2012:489830.
153. Kim J, Inoue K, Ishii J, Vanti WB, Voronov SV, Murchison E, et al. A MicroRNA feedback circuit in midbrain dopamine neurons. Science. 2007;317(5842):1220–4.
154. Hwang D-Y, Ardayfio P, Kang UJ, Semina EV, Kim K-S. Selective loss of dopaminergic neurons in the substantia nigra of Pitx3-deficient aphakia mice. Brain Res Mol Brain Res. 2003;114(2):123–31.
155. Long JM, Ray B, Lahiri DK. MicroRNA-339-5p down-regulates protein expression of β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) in human primary brain cultures and is reduced in brain tissue specimens of Alzheimer disease subjects. J Biol Chem. 2014;289(8):5184–98.
156. Kim Y-K, Kim VN. Processing of intronic microRNAs. EMBO J. 2007;26(3):775–83.
157. Hardy J, Lewis P, Revesz T, Lees A, Paisan-Ruiz C. The genetics of Parkinson's syndromes: a critical review. Curr Opin Genet Dev. 2009;19(3):254–65.
158. Sotiriou S, Gibney G, Baxevanis AD, Nussbaum RL. A single nucleotide polymorphism in the 3′UTR of the SNCA gene encoding alpha-synuclein is a new potential susceptibility locus for Parkinson disease. Neurosci Lett. 2009;461(2):196–201.
159. Doxakis E. Post-transcriptional regulation of alpha-synuclein expression by mir-7 and mir-153. J Biol Chem. 2010;285(17):12726–34.
160. Prajapati P, Sripada L, Singh K, Bhatelia K, Singh R, Singh R. TNF-α regulates miRNA targeting mitochondrial complex-I and induces cell death in dopaminergic cells. Biochim Biophys Acta. 2015;1852(3):451–61.
161. Junn E, Lee K-W, Jeong BS, Chan TW, Im J-Y, Mouradian MM. Repression of alpha-synuclein expression and toxicity by microRNA-7. Proc Natl Acad Sci U S A. 2009;106(31):13052–7.
162. McDermott CJ, Shaw PJ. Diagnosis and management of motor neurone disease. BMJ. 2008;336(7645):658–62.
163. Haramati S, Chapnik E, Sztainberg Y, Eilam R, Zwang R, Gershoni N, et al. miRNA malfunction causes spinal motor neuron disease. Proc Natl Acad Sci U S A. 2010;107(29):13111–6.
164. Campos-Melo D, Droppelmann CA, He Z, Volkening K, Strong MJ. Altered microRNA expression profile in Amyotrophic Lateral Sclerosis: a role in the regulation of NFL mRNA levels. Mol Brain. 2013;6:26.
165. Williams AH, Valdez G, Moresi V, Qi X, McAnally J, Elliott JL, et al. MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. Science. 2009;326(5959):1549–54.
166. McCarthy JJ. MicroRNA-206: the skeletal muscle-specific myomiR. Biochim Biophys Acta. 2008;1779(11):682–91.
167. Russell AP, Wada S, Vergani L, Hock MB, Lamon S, Léger B, et al. Disruption of skeletal muscle mitochondrial network genes and miRNAs in amyotrophic lateral sclerosis. Neurobiol Dis. 2012;49C:107–17.
168. Zuccato C, Valenza M, Cattaneo E. Molecular mechanisms and potential therapeutical targets in Huntington's disease. Physiol Rev. 2010;90(3):905–81.
169. Imarisio S, Carmichael J, Korolchuk V, Chen C-W, Saiki S, Rose C, et al. Huntington's disease: from pathology and genetics to potential therapies. Biochem J. 2008;412(2):191–209.
170. Seredenina T, Luthi-Carter R. What have we learned from gene expression profiles in Huntington's disease? Neurobiol Dis. 2012;45(1):83–98.
171. Savas JN, Makusky A, Ottosen S, Baillat D, Then F, Krainc D, et al. Huntington's disease protein contributes to RNA-mediated gene silencing through association with Argonaute and P bodies. Proc Natl Acad Sci U S A. 2008;105(31):10820–5.

172. Lee S-T, Chu K, Im W-S, Yoon H-J, Im J-Y, Park J-E, et al. Altered microRNA regulation in Huntington's disease models. Exp Neurol. 2011;227(1):172–9.
173. Ghose J, Sinha M, Das E, Jana NR, Bhattacharyya NP. Regulation of miR-146a by RelA/NFkB and p53 in STHdh(Q111)/Hdh(Q111) cells, a cell model of Huntington's disease. PLoS One. 2011;6(8), e23837.
174. Jin J, Cheng Y, Zhang Y, Wood W, Peng Q, Hutchison E, et al. Interrogation of brain miRNA and mRNA expression profiles reveals a molecular regulatory network that is perturbed by mutant huntingtin. J Neurochem. 2012;123(4):477–90.
175. Sinha M, Ghose J, Das E, Bhattarcharyya NP. Altered microRNAs in STHdh(Q111)/Hdh(Q111) cells: miR-146a targets TBP. Biochem Biophys Res Commun. 2010;396(3):742–7.
176. Gaughwin PM, Ciesla M, Lahiri N, Tabrizi SJ, Brundin P, Björkqvist M. Hsa-miR-34b is a plasma-stable microRNA that is elevated in pre-manifest Huntington's disease. Hum Mol Genet. 2011;20(11):2225–37.
177. Sinha M, Ghose J, Bhattarcharyya NP. Micro RNA -214,-150,-146a and-125b target Huntingtin gene. RNA Biol. 2011;8(6):1005–21.
178. Kocerha J, Xu Y, Prucha MS, Zhao D, Chan AWS. microRNA-128a dysregulation in transgenic Huntington's disease monkeys. Mol. Brain. 2014;7:46.

Chapter 9
microRNAs in Cerebrovascular Disease

Ondřej Volný, Linda Kašičková, Dominika Coufalová, Petra Cimflová, and Jan Novák

Abstract Cardiovascular diseases are major causes of morbidity and mortality in developed countries. Cerebrovascular diseases, especially stroke, represent major burden of disability and economy impact. Major advances in primary and secondary prevention and therapy are needed in order to tackle this public health problem. Our better understanding of pathophysiology is essential in order to develop novel diagnostic and therapeutic tools and strategies. microRNAs are a family of important post-transcriptional regulators of gene expression and their involvement in the pathophysiology of cerebrovascular diseases has already been reported. Moreover, microRNAs may represent above-mentioned potential diagnostic and therapeutic tools in clinical practice. Within this chapter, we briefly describe basic epidemiology, aetiology and clinical manifestation of following cerebrovascular diseases: extracranial carotid atherosclerosis, acute stroke, intracranial aneurysms and cerebral arterio-venous malformations. Further, in each chapter, the current knowledge about

O. Volný, M.D.
Department of Neurology, St. Anne's University Hospital and Faculty of Medicine, Masaryk University, Pekarska 53, Brno 656 91, Czech Republic

Department of Anatomy, Faculty of Medicine, Masaryk University, Kamenice 5, Brno 625 00, Czech Republic

International Clinical Research Center, St. Anne's University Hospital, Pekarska 53, Brno 656 91, Czech Republic
e-mail: 214565@mail.muni.cz; Ondrej.Volny@memorix.cz

L. Kašičková
Department of Neurology, St. Anne's University Hospital and Faculty of Medicine, Masaryk University, Pekarska 53, Brno 656 91, Czech Republic

Department of Anatomy, Faculty of Medicine, Masaryk University, Kamenice 5, Brno 625 00, Czech Republic
e-mail: 381169@mail.muni.cz

D. Coufalová
Department of Anatomy, Faculty of Medicine, Masaryk University, Kamenice 5, Brno 625 00, Czech Republic

International Clinical Research Center, St. Anne's University Hospital, Pekarska 53, Brno 656 91, Czech Republic
e-mail: 381169@mail.muni.cz

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_9

the involvement of specific microRNAs and their potential use in clinical practice will be summarized. More specifically, within the subchapter "miRNAs in carotid atherosclerosis", general information about miRNA involvement in atherosclerosis will be described (miR-126, miR-17-92, miR-155 and others) with special emphasis put on miRNAs affecting carotid plaque progression and stability (e.g. miR-145, miR-146 or miR-217). In the subchapter "miRNAs in acute stroke", we will provide insight into recent knowledge from animal and human studies concerning miRNA profiling in acute stroke and their expression dynamics in brain tissue and extracellular fluids (roles of, e.g. let-7 family, miR-21, miR-29 family, miR-124, miR-145, miR-181 family, miR-210 and miR-223). Subchapters dealing with "miRNAs and AV malformations" and "miRNAs and intracranial aneurysms" will focus on miR-21, miR-26, miR-29 family and miR-143/145.

Keywords microRNA • Carotid atherosclerosis • Ischemic stroke • Intracranial aneurysms • Cerebral arterio-venous malformations

Carotid Atherosclerosis

Epidemiology of Carotid Atherosclerosis

Atherosclerosis represents a complex arterial disease characterized by vascular wall inflammation and remodelling in the end resulting in the creation of atherosclerotic plaques [1]. According to the site of plaques occurrence, atherosclerosis clinically presents as coronary artery disease (CAD), carotid atherosclerosis (Fig. 9.1) or peripheral artery disease (PAD) [2]. Carotid arteries represent one of the specific places of plaques accumulation, especially concerning the ostium and bifurcation of common carotid artery, probably due to alteration of blood flow at these locations [3]. Accumulation of atherosclerotic plaques in carotid arteries

P. Cimflová, M.D.
Department of Radiology, St. Anne's University Hospital and Faculty of Medicine, Masaryk University, Pekarska 53, Brno 656 91, Czech Republic
e-mail: petra.cimflova@fnusa.cz

J. Novák, M.D. (✉)
2nd Department of Internal Medicine, St. Anne's University Hospital and Faculty of Medicine, Masaryk University, Pekarska 53, Brno 656 91, Czech Republic

Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Kamenice 5, Brno 62500, Czech Republic

Department of Physiology, Faculty of Medicine, Masaryk University, Brno 62500, Czech Republic
e-mail: jnmedved@seznam.cz

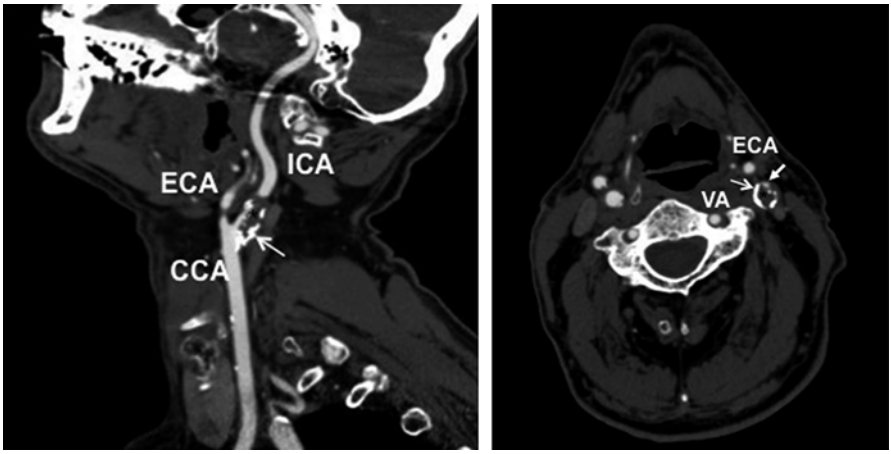

Fig. 9.1 Atherosclerotic plaque in the left internal carotid artery, curved multiplanar reconstruction (MPR). Sagittal (*left*) and axial (*right*) CT angiography scans of the neck demonstrating >90 % narrowing of the internal carotid artery behind the common carotid artery bifurcation. Atherosclerotic plaque is represented by a hypodense area in the arterial lumen (*black star*), *open arrows* point to the plaque calcifications (the most apparent hyperdense part; on the axial scan forming a hyperdense rim). *White arrow* on the axial scan (*right*) shows the residual vascular lumen (diminished by mass of the plaque). *CCA* common carotid artery, *ECA* external carotid artery, *ICA* internal carotid artery, *VA* vertebral artery

affects significantly blood supply to the brain and predisposes patients to fainting, syncope and possibly to ischemic stroke. Based on data of Framingham cohort study, the reported prevalence of carotid artery atherosclerosis (defined as stenosis >50 % evaluated by carotid ultrasound) in the study population was 7 % in women and 9 % in men [4–6]. In term of stroke aetiology, the atherosclerotic aetiology accounts for 10–15 % of ischemic strokes and soon revelation of carotid atherosclerosis represents a risk factor for future stroke development [7].

Clinical Evaluation of Carotid Atherosclerosis

Carotid stenosis can be evaluated by various non-invasive methods [8, 9]:

- *B-mode ultrasound* (parameters as intima-media thickness (IMT), echogenic quality and 3D volume measurement of plaque characteristics)
- *Transcranial Doppler* (microemboli detection used as a marker of plaque instability; detection of intracranial carotid stenosis)
- *Computed tomography angiography/CTA* (assessment of arterial stenosis and basic plaque characteristics)
- *Magnetic resonance imaging/MRI* (evaluating the intraplaque haemorrhage and presence of a fibrous cap, both representing radiological markers of plaque vulnerability)

- *Positron emission tomography/PET* (research imaging technique used for evaluation of vascular wall inflammation)

Periodical carotid plaque evaluation represents a tool for therapy monitoring and carotid plaque evolution. The most important is a proper treatment of vascular risk factors (hypertension, diabetes, dyslipidemia, smoking cessation, etc.), specifically, the therapy of dyslipidemia is typically led by statins which lower serum cholesterol levels, reduce subclinical inflammation inside the plaque, and promote intraplaque stabilization independently of cholesterol lowering as well [10].

Pathophysiology of Atherosclerosis and Roles of miRNAs

From the pathophysiological point of view, atherosclerosis represents a complex process of arterial wall inflammation and remodelling that includes activation of endothelial cells (ECs) by various external stimuli, e.g. disrupted blood-flow [3] or oxidized LDL (oxLDL) presence [11], which further leads to activation/proliferation of vascular smooth muscle cells (VSMCs), vascular wall fibroblasts and also other cell types, such as platelets, adipocytes or macrophages (and their altered form "foam cells") [1, 11]. All above-mentioned processes results in the end in the creation of atherosclerotic plaques. Predominantly, atherosclerotic plaques are formed at places exposed to disrupted blood flow, in vivo specifically in the aortic arch and arterial bifurcations [12]. Recent advances in our understanding of atherosclerosis pathophysiology revealed that shear stress significantly affects the gene expression of EC exposed to either high or low shear stress, and miRNAs seem to participate even in the flow-regulation of atherosclerosis [12, 13].

Roles of miRNA in atherosclerosis include mainly (a) regulation of gene expression in all aforementioned cells and (b) miRNAs serve as mediators of cell-to-cell communication [13, 14]. From the atherosclerotic point of view, miRNAs (also termed "athero-miRs") can be divided to several groups concerning their individual characteristics—to mechano-responsive or mechano-irresponsive (depending on the response to shear-stress) or to proatherogenic, antiatherogenic or ambigual (according to their effects on atherosclerosis progression) [15]. Providing the complex overview of miRNAs involved in atherosclerosis is not the main goal in this subchapter—here we will provide just a brief summary of the roles of main atheromiRs, i.e. miR-17-92 cluster, miR-126, miR-143/145 cluster and miR-155, and then we will specifically focus on human studies dealing with carotid atherosclerosis.

AtheromiRs

Concerning the complex pathophysiology of atherosclerosis, from all potential miRNAs involved, we should definitely mention ,widespread miR-17-92 cluster [16], endothelium-specific miR-126 [17], vascular-smooth muscle, cell enriched

miR-143/145 cluster [18, 19], and also immunity-related miR-155, that is even tightly connected to macrophages function [20].

Members of miR-17-92 cluster (i.e. miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a) were shown to be downregulated after the exposure of endothelial cells to laminar (≈stable) flow [21], which makes miR-17-92 proatherogenic—we can imagine that by downregulating miR-17-92, endothelium somehow "protects itself" from miR-17-92 effects. miR-17-92 was shown to target Krüppel-like factor (KLF)-2 and KLF-4 and this targeting promoted endothelial inflammation—on the other hand, if miR-17-92 was inhibited, it reduced the expression of proinflammatory markers [22]. Moreover, on the contrary to tumour angiogenesis that is promoted by miR-17-92 increase [23, 24], endothelial sprouting and neovascularization are promoted if miR-17-92 cluster activity is decreased [23, 24] and this may potentially contribute to restenosis occurrence. Concerning other general roles of miR-17-92 cluster in the regulation of cell cycle and apoptosis [25], more studies are needed to shed a light on the precise functions of this cluster in atherosclerotic process; however, inhibition of miR-17-92 cluster may generally be considered atheroprotective.

Endothelial specific miR-126 (and its passenger strand miR-126*) are both known to be highly expressed in endothelium and they are crucial for vascular integrity and the vessel development (by targeting PIK3R2, Spred-1 [17, 26], VEGF [27] or, e.g. by affecting angiopoietin signalling [28]). Their upregulation in endothelial cells generally seems to be atheroprotective. By targeting vascular cell adhesion molecule (VCAM)-1 miR-126 attenuates endothelial inflammation [29]. Similarly, miR-126 packaged in apoptotic bodies enhances endothelial recovery through upregulation of CXCL12 (particularly by inhibiting RGS16, CXCL12 inhibitor [30]), and miR-126* keeps endothelial proliferative reserve by targeting DLK1 [31]. Moreover, circulating levels of miR-126 were shown to be decreased in patients with coronary atherosclerosis and diabetes, which may contribute to the pathophysiology of both diseases [32, 33]. On the other hand, miR-126 transfer into VSMCs was shown to promote VSMCs turnover, thus suggesting a potentially negative role in vascular remodelling [34]. miR-126 functions thus need to be evaluated strictly in the tissue (or cell)-dependent manner.

Focusing now on VSMCs, miR-143/145 should be mentioned. To fulfil their functions in the vessel tone regulation, VSMCs need to remain in so-called *contractile* (or *quiescent*) phenotype; during various pathologies, the phenotypic switch to "synthetic" (or "proliferative") phenotype occurs interfering with VSMCs normal function [35]. miR-143/145 was shown to be one of the crucial regulators of the phenotypic switch—increased levels of miR-143/145 are needed in VSMCs to remain in "contractile" phenotype [18, 36]. Knockout of miR-143/145 leads to incomplete differentiation of VSMCs and results, e.g. in aneurysm development [36]. Interestingly, it was shown that miR-143/145 levels are increased in ECs under the laminar flow [37] and moreover, miR-143/145 is transferred from ECs into VSMCs in order to keep them in "contractile" phenotype [38]. miR-143/145 targeting thus definitely represents a potential therapeutic target in the treatment of atherosclerosis for the future.

Lastly, if talking about atherosclerosis, macrophages need to be mentioned together with miR-155. miR-155 represents a master inflammation-miRNA and the important regulator of the immune system functions [20]. It was repeatedly shown that miR-155 knockout or inhibition in experimental models of atherosclerosis led to reduction of atherosclerotic plaques size and decreased macrophage accumulation [39, 40]. Via targeting of endothelial NO-synthase (eNOS), miR-155 further disrupts endothelial relaxation, which also contributes to the pathophysiology of atherosclerosis [41]. Interestingly, circulating (particularly plasmatic) levels of miR-155 were shown to be increased in experimental models of atherosclerosis [42] and miR-155 was shown as one of the most abundant miRNAs contained within LDL particles. This may partly explain LDL proinflammatory effects [43]. On the contrary, also antiatherogenic effects or miR-155 were described—e.g. absence of miR-155 in the haematopoetic cells in the hyperlipidemic mice increased atherosclerotic plaque formation [44]. Moreover, in human subjects with CAD, circulating levels of miR-155 are reduced [32]. More information about the conflicting role of miR-155 within atherosclerosis development can be found in the recent study performed by Ma et al. [45].

miRNAs in Carotid Atherosclerosis in Humans

Human studies dealing with carotid atherosclerosis mostly determined expression of miRNAs from carotid atherosclerotic plaques obtained during endarterectomy of carotid arteries in patients with symptomatic stenosis [46–48]. Comparison of carotid plaques miRNA expression revealed a set of differentially expressed miRNAs compared to miRNA expression in normal carotid arteries and within this set, miR-21, miR-34a, miR-146a, miR-146b-5p and miR-210 were found to be upregulated [47]. In the very interesting study by Cipollone et al., the subset of five miRNAs (miR-100, miR-127, miR-145, miR-133a and miR-133b) were further shown to be increased in patients after stroke compared to patients with plaques but without stroke, thus predicting higher plaque instability [46]. In the agreement with Cipollone results, miR-145 expression was shown to be higher in carotid plaques from patients with hypertension [48], who are more prone to develop stroke if hypertension is not properly treated. Higher carotid plaque expression of miR-145 may thus be considered as a risk factor for plaque instability [46, 48]. Interestingly, higher circulating miR-145 levels were shown [49] and at the same time abolished [50] as a potential stroke biomarker (as described further).

Focusing more on circulating miRNAs, levels of miR-21 and miR-221 were shown to be higher in patients with carotid atherosclerosis [50] and urinary levels of miR-29b were shown to correlate with cIMT in patients with type 2 diabetes mellitus (T2DM) [51]. All three miRNAs may thus serve as potential markers of carotid atherosclerosis reflecting the disease severity [50, 51].

Lastly, we would like to point out very interesting role of miR-217 described in the senescence of ECs (using human umbilical vein ECs, aortic ECs and coronary ECs together with endarterectomy samples) [52]. miR-217 was shown to be expressed in human carotid plaques and it was shown to regulate the expression of silent information regulator 1 (Sirt1)—as the cells were ageing, miR-217 levels were increasing with concomitant decrease in Sirt1 levels, a phenomenon known to occur during ageing. Blockade of miR-217 reversed effect of ageing and increased angiogenic activity. miR-217 antagonism may thus represent very interesting strategy to reduce endothelial senescence and prevent the development of age-related diseases.

Summary

Both atherosclerosis in general and particularly carotid atherosclerosis represent risk factor for stroke development. Studies using endarterectomy and plasma/urine samples to reveal biomarkers of plaque instability or disease severity definitely indicate that miRNAs may serve not only as potential diagnostic but also therapeutic targets in carotid atherosclerosis. Further studies on larger cohorts and involving patients with various comorbidities are needed as well as studies using animal models of plaque instability, to reveal the true potential of miRNAs in the carotid atherosclerosis for future clinical practice.

Ischemic Stroke

Epidemiology of Ischemic Stroke

Stroke represents a major cause of disability and the second leading cause of death worldwide (after coronary artery disease), with an incidence of about 17 million per year. It affects both the elderly and the young (in 2010, 31 % of strokes affected adults under 65; more than 83,000 children and youths under 20 have had strokes). Improvements in primary prevention and lifestyle changes have led to a decreased incidence of age-adjusted stroke. Nevertheless, the overall number of strokes has been increasing and is expected to accelerate over the coming decades because of the population ageing. It is predicted that stroke will account for 6.2 % of the total burden of illness in developed countries by 2020. Without major advances in primary and secondary prevention, acute stroke management and treatment, and in post-stroke rehabilitation, the burden and cost of this disease will considerably increase [53–55]. Stroke can be classified as ischemic or haemorrhagic. Ischemic stroke can be either transient ischemic attack (TIA) or cerebral infarction. Haemorrhagic strokes can be either intracerebral or subarachnoid. Figure 9.2 shows the computed tomography (CT) differences between acute ischemic and haemorrhagic strokes.

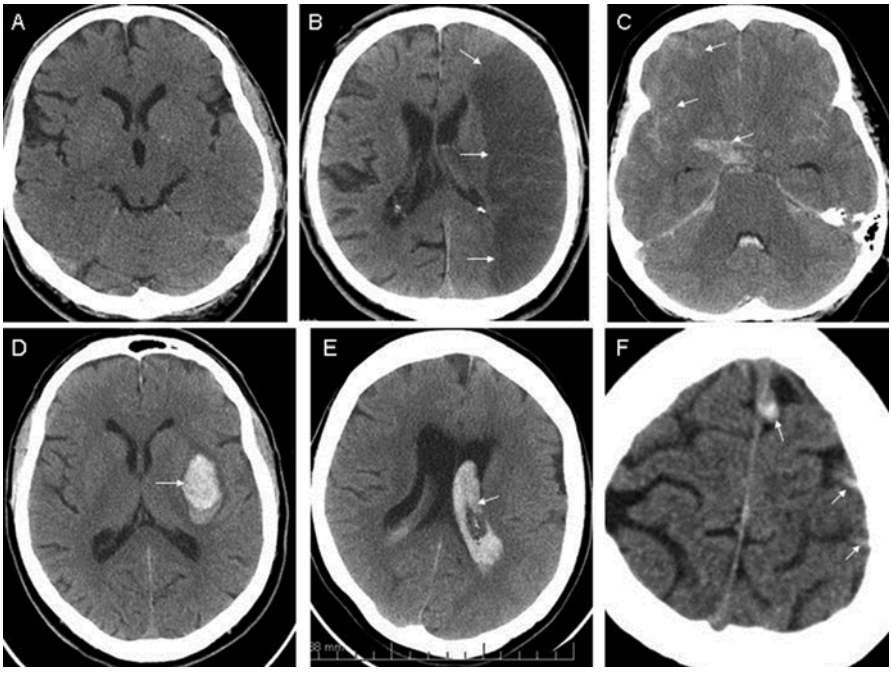

Fig. 9.2 Ischemic and haemorrhagic stroke on CT. (**a**) Normal CT (typical baseline finding in patients with acute ischemia); (**b**) acute ischemia 24 h after stroke onset (hypodense area marked by *white arrows*); (**c**) subarachnoid haemorrhage (hyperdense signal marked by *white arrows*); (**d**) intracerebral haemorrhage (ICH); (**e**) intraventricular haemorrhage; (**f**) cortical vein/dural sinus thrombosis (hyperintensities represents acute clots in cortical vein and superior sagittal sinus)

Brief Aetiology and Pathophysiology of Ischemic Stroke and Intracerebral Haemorrhage

Ischemic strokes represent at least 85 % of all strokes. Sometimes, the cerebral infarctions with a small deficit are called "minor strokes". Cerebral ischemia represents a consequence of arterial or arteriolar occlusion leading to the blood flow reduction. Possible ischemic stroke mechanisms (aetiologies) include ischemic strokes of atherotrombotic (30 %), lacunar (20 %) or cardioembolic origin (30 %); of other known aetiology, e.g. arterial dissection, vasculitis, hemodynamic infarctions, thrombophilia, cortical vein or dural sinus thrombosis (5–10 %); and 5–10 % are strokes of unknown cause termed cryptogenic strokes [54, 56].

It is important to mention that therapeutic and preventive strategies depend on the proper diagnosis of the stroke cause. Typical example is an indication for anticoagulation versus antiplatelet therapy (secondary prevention) in patients with cardioembolic versus non-cardioembolic strokes. Identification of the exact cause is also important for prognosis and treatment of additional risk factors.

Intracerebral haemorrhages (ICHs) are divided in the primary and secondary. Primary ICHs are caused by a rupture of cerebral artery/arteriole, and are typically associated with uncontrolled hypertension. Based on the localization apparent on neuroimaging methods, they can be divided into two main types: typical and atypical. The typical ICH is located in deep brain structures supplied by perforating arteries. The atypical ICHs are located more superficially (e.g. lobar haemorrhages). Atypical ICHs (20 % of all ICH) are usually secondary to medical conditions different to hypertension, such as coagulopathies, arterio-venous malformations, other vasculopathies, and tumours.

From clinical point of view, presentation of ICH is very similar to acute ischemic stroke, and therefore ICHs cannot be differentiated from acute ischemia based only on clinical examination. ICH manifestation corresponds to its location and volume, and also to the bleeding progression. Because treatments of ischemic and haemorrhagic strokes differ, brain CT or MRI are essential for differential diagnosis and treatment decision-making [57–60].

microRNA in Acute Ischemic Stroke

There are numerous roles of miRNAs in acute ischemic stroke—e.g. intracellular miRNAs participate in pathophysiology of ischemic stroke via affecting the target genes expression involved in atherosclerosis, angiogenesis and/or neuroinflammation [61]; extracellular miRNAs serve as mediators of cell-to-cell communication affecting pathophysiology and course of the disease. Molecules released into the extracellular space might represent clinically valuable biomarkers. It was shown that almost any ischemic conditions (coronary artery disease, peripheral artery disease) lead to alteration in circulating miRNA levels [62]. The tissue-specific miRNAs are released into the circulation after cell damage or death, and thus indirectly indicate the organ damage [63, 64]. Since the levels of circulating miRNAs are not random and exact mechanisms of miRNA secretion/excretion still need to be clarified, these miRNAs may even reflect the course of disease and bring to clinicians information regarding patients prognosis and outcome (e.g. in myocardial infarction, specific miRNA profile may predict development of heart failure [65]; in stroke, miRNA profiles were shown to be predictive in stroke recurrence [66], etc.).

Within the field of ischemic stroke, miRNAs have been studied both in different animal models and human stroke populations. In terms of miRNAs detection both principal approaches, i.e. studying the expression profiles of many miRNAs at the same time (“miRNA profiling”), as well as candidate-miRNAs approach (i.e. focusing more deeply on a subset of miRNAs, their possible targets and biological roles) have already been employed.

Within the following subchapter, we will focus on profiling studies giving a rationale for further miRNA studies in the field of ischemic stroke.

Animal Models of Stroke

Experimental stroke models used in following studies comprise mainly of cell culture (varying from generally accessible cell cultures such as HEK cells, N2A cells, to the primary cultured cortical neurons) of oxygen-glucose deprivation (OGD) simulating hypoxic-ischemic conditions by restriction of oxygen and glucose or pure hypoxia (e.g. for 30 to 60 min). Otherwise the H_2O_2 application responsible for simulating the reactive oxygen species (ROS) specific damage has been induced and studied. In studies focused on the roles of progenitor cells after acute ischemia, the subventricular zone (SVZ) cells has been isolated and studied in hypoxic-ischemic conditions.

In animal model studies, models of the middle cerebral artery occlusion (MCAO) have been typically used but with different approaches to the induction of occlusion (usually lasting from 60 to 120 min followed by varying periods of reperfusion and sampling). The way of MCAO technique has been also highly variable (using electro-coagulation, embolic techniques or complete mechanical obstruction, etc.). In terms of animals, spontaneously hypertensive rats (SHR), Wistar rats, Sprague-Dawley rats or C57BL/6J mice have been used.

In human studies, there is no possibility to simulate experimental stroke, thus only patients admitted to hospitals with stroke symptoms might be involved in miRNA sampling from different bodily fluids. So far, only the blood and its derivatives, and/or cerebrospinal fluid have been used in human stroke research.

Profiling microRNA Studies in Acute Ischemic Stroke

Up to date several miRNA profiling studies were performed using both animal model of stroke [67–72] and human stroke patients [73–78], in the animal studies using either brain tissue or blood or both; in the human studies using bodily fluids of patients after ischemic event.

Animal Profiling Studies

The very first miRNA profiling in animal stroke model was performed by Jeyaseelan et al. in 2008. Using MCAO model, they performed screen for 236 various miRNAs in both blood and brain at two different time points (20/106 and 25/82 differentially expressed miRNAs at 24 and 48 h in blood/brain were identified). Moreover, the expression of following miRNAs was detected for the first time in the rat brain: miR-126, miR-129, miR-129*, miR-145, miR-181a/b/c and miR-185.

Focusing on the specific group of miRNAs the authors confirmed several targets: laminin-1 and integrin-1 as targets of miR-124, VSNL1 (neuronal calcium sensor)

as a target of miR-290 (and possibly also miR-124a); aquaporin (AQP4) as a target of miR-30a-3p and miR-383, and matrix metalloproteinase 9 (MMP9) as a target of miR-132 and miR-664 [69]. In their following study, they focused on miRNAs expression in later phases of stroke (48–168 h after ischemic stroke) and identified: miR-21, miR-142-3p, miR-142-5p and miR-146a to be increased and miR-196a/b/c, miR-224 and miR-324-3p to be decreased as compared to the acute phase. Moreover, levels of miR-206, miR-290, miR-291a-5p and miR-30c-1* positively correlated with infarct volume [70].

Further, Dharap and colleagues used SHR and extended findings from the first study of Jeyaseelan by sacrificing animals at five different time points (3, 6, 12, 24 and 72 h). It was shown that 49 miRNAs were dysregulated after transient MCAO, 24 miRNAs were upregulated and 25 showed downregulation at least one of these time points. Other subsets of miRNAs were detected at two or more time points showing that expression of some miRNAs (e.g. miR-140, miR-145, miR-260, miR-292-5p) tended to increase and of other miRNAs (e.g. miR-376-5p, miR-153) tended to decrease over time, while other miRNAs were altered only transiently (e.g. miR-29c) or permanently without the specific increase or decrease (e.g. miR-344-3p).

The authors further focused on miR-145 and its target superoxide dismutase (SOD2) using antagomir against miR-145 showing that inhibition of miR-145 led to SOD2 upregulation and consequently protected the neurons from cell death [67]. Similarly to Dharap, Gubern et al. in 2013 also proved the expression change of 32 miRNAs during acute phase of stroke (30 min and 6 h) and late stroke phases (7 and 14 days). Their group further focused on miR-347 and its targets (Acsl4, Bnip31 and Phyhip) showing its importance in regulation of neuronal cell death [68].

Compared to previously mentioned studies, Selvamani et al. [71] focused on stroke from slightly different point of view—studying effect of age and sex on miRNA expression (miR-127-3p, miR-335, miR-543, miR-139-5p, miR-33a, miR-338-3p, miR-222, miR-15b, miR-92a, miR-424, miR-495 and miR-33) in blood and brain at day 2 and 5 after the MCAO. Interestingly, on the day 2, differences in miRNA levels were affected mainly by age, and on the day 5 mainly by sex showing the need to keep in mind the confounding factors.

Animal profiling studies definitely created a great background for the studies mentioned further by identifying a wide range of miRNAs altered in brain tissue after stroke. These studies showed that a large number of miRNAs was altered in rat brain after ischemic event and that this set was not completely the same as the set of blood identified miRNAs. Nowadays, it has been revealed that after stroke, also other organs than brain are involved in the complex body reaction affecting patient outcome. Thus identification of other miRNAs involved, e.g. in inflammation, or being other organ specific (e.g. finding of liver specific miR-122 to be increased in blood after stroke) may further stimulate the research in order to better understand the complex effect of stroke on the organism.

Human Profiling Studies

Unlike the animal profiling studies, where brain tissue is available and enable much deeper focus on molecular biology of stroke, only bodily fluids are available in ischemic stroke in humans for miRNA analysis.

One of the first attempts to identify circulating miRNAs in peripheral blood of 19 patients after stroke was performed by Tan et al. in 2009. Relatively young patients (aged 19–49 years) were enrolled and from 836 miRNAs studied in total, 157 were found to be dysregulated after stroke (138 upregulated, 19 downregulated). Out of these let-7f, miR-126, -1259, -142-3p, -15b, -186, -519e and -768-5p were consistently downregulated in all patients independently of the stroke subtype (according to TOAST classification).

Interestingly, miRNA clustering revealed different expression patterns in patients with good and poor clinical outcome defined as modified Rankin scale (mRS) ≤ 2 versus mRS >2. Although being a small-sized study enrolling only young individuals, the very promising results definitely identified miRNAs potentially useful as biomarkers of acute stroke and thus much larger studies were performed by the same group in 2013 [76] and 2014 [74].

In the first mentioned study, authors studies no-risk or low-risk patients with stroke, to avoid a potential effect of atherosclerosis, hypertension or diabetes on circulating miRNA profiles and identified 293 miRNAs out of whose 21 miRNAs presented with similar expression in all stroke samples [76]. Four of these, miR-25*, -34b, -483-5p and miR-498, were shown to be downregulated in all cases.

In the latter mentioned study, the authors involved two groups of stroke patients: relatively young (≈45 years) and older cohort (≈60 years) after stroke. The latter cohort displayed a number of age-related comorbidities, which also was a reason to involve healthy control (≈40 years) and patients with metabolic syndrome without the history of stroke as two control cohorts [74]. Out of 314 miRNAs studied, downregulation was observed in 58 and upregulation in 47 miRNAs in acute (24, 48 h up to 7 days) or late phase (from 2 months to 2 years) of stroke. Sixty-three miRNAs were found to be altered at both phases and five upregulated miRNAs, miR-27a*, miR-125b-2*, miR-422a, miR-488 and miR-627, showed high AUCs for patients after stroke, compared to those with metabolic syndrome. Moreover, authors showed that clustering of 32 miRNAs enabled distinguishing among individual stroke subtypes according to TOAST classification.

Another profiling after stroke was performed by Wang et al. [78]. This study group focused on early stroke detection using MRI in 136 stroke patients within 24 h from stroke onset. Based on the MRI finding, MRI(+) and MRI(−) stroke patients were distinguished and profiles were taken for these patients together with 116 healthy subjects. Altogether, 42 miRNAs were chosen for further confirmation and out of these, 13 miRNAs were confirmed as upregulated and 4 miRNAs as being downregulated. miR-106b-5p and miR-4306 showed gradual expression and being the lowest in controls, higher in MRI(−) and the highest in MRI(+) patients. Contrarily, miR-320e and miR-320d showed opposite trend, i.e. being downregu-

lated in MRI(−) and more downregulated in MRI(+) patients, thus being identified as potential biomarkers of acute stroke.

One of the very recent profiling studies of human serum was performed by Li et al. This study identified 115 differentially expressed miRNAs in ischemic stroke patients. Out of whose miR-32-3p, miR-106-5p, miR-1246 and miR-532-5p were identified as potential stroke biomarkers [79].

Another attempt to study specific roles of miRNAs in stroke was performed by Jickling et al. In this study the authors determined miRNA profiles from peripheral blood mononuclear cells, not from plasma or serum [73]. This different approach enables to study leukocytes expression profiles and reveal a potential interconnection between immunity, inflammation and stroke. In conclusion miR-122, miR-148a, let-7i, miR-19a, miR-320d and miR-4429 were decreased and miR-363 and miR-487b increased in the cells of patients with stroke and thus the potential regulation of leukocyte adhesion, extravasation and thrombi formation was proposed [73].

Most recently, Sorensen et al. published interesting paper identifying potential miRNA biomarkers of acute stroke not only from blood but also using the cerebrospinal fluid [75]. At the time of this manuscript preparation, only abstract of this study was available. The authors identified miR-151-3p and miR-140-5p to be upregulated and miR-18b-5p to be downregulated in blood after stroke and miR-523-3p was identified in more than 50 % of patients and in none of the controls in the cerebrospinal fluid. These results, together with results from other research groups mentioned above or further clearly demonstrate a potential of miRNAs as biomarkers of acute ischemic stroke and the need for complex studies taking into account not only the traditional cardiovascular and metabolic risk factors, sex, age but also using control cohorts with other neurological disorders that can resemble stroke.

Candidate microRNA Studies

Following section summarizes up-to-date information regarding animal and human studies in acute stroke with the special emphasis put on the let-7 family, miR-21, miR-29 family, miR-124, miR-145, miR-181 family, miR-210 and miR-223. Roles of other miRNAs are briefly summarized in the last subsection. Overview of below described miRNAs is provided in Tables 9.1 and 9.2, detailed description can be found further in the text.

let-7 Family

let-7 family represents a group of nine miRNAs termed let-7a to let-7i. Being one of the first miRNAs even described with important functions in *C. elegans* developmental timing [111], its functions in humans has been extensively studied and except of participating in tumorigenesis, angiogenesis and immunity, let-7 family

Table 9.1 Overview of the targets and roles of the main miRNAs involved in stroke pathogenesis

miR	Target	Model	Function in stroke	References
Let-7 family	–	–	Involved in neurogenesis and neuronal differentiation. Downregulated in stroke	[80]
	IGF-1 signalling	–	Application of antagomir against let-7f led to increased neuroprotection	[81]
miR-21	Fas-Ligand	MCAO Wistar, CN, HEK-293	Expression of miR-21 is increased after MCAO. In OGD cultured cortical neurons, miR-21 levels are unchanged. Introduction of miR-21 into HEK cells caused downregulation of Fas-Ligand. Potential therapeutic target	[82]
	Bcl-2	N2A NBLC	Increased expression of miR-21 by reoxygenation after OGD in N2A cells had antiapoptotic effect	[83]
miR-29 family	BH3-genes	MCAO, C57BL/6, hippocampal neural cells	miR-29b and miR-29c are downregulated after stroke and this can be prevented by oral supplementation of α-tocotrienol. Stereotactic injection of miR-29b improved brain function and lowered infarct size. Moreover, miR-29b transfection into cell lines protected cells from cell death	[84]
	DNMT3a	MCAO, SHR PC12 cells	MCAO in adult rat caused downregulation of miR-29c, which promotes ischemic damage to brain via DNMT1a induction. Substitution of miR-29c improves survival and neuronal damage	[85]
	Bcl2L2	MCAO, Wistar, CN	miR-29b is upregulated after ischemic insult and promotes neuronal cells death by targeting Bcl2L2 in MCAO model	[86]
	Birc2 (Bak1)	FNS in SDR	Fastigial nucleus stimulation (FNS) is known to reduce brain damage and its effect is partly mediated by downregulation of miR-29c. Application of FNS prior to ischemic insult had neuroprotective effect mediated by miR-29c	[87]
	–	MCAO, C57BL/6 J, CN	Study of miRNA expression in salvageable ischemic penumbra: *miR-29b-2*, -19b, 339-5p and miR-341 are dysregulated and they possibly participate on regulation of gene expression in this area	[88]

(continued)

Table 9.1 (continued)

miR	Target	Model	Function in stroke	References
miR-124	VSNL1	MCAO, SDR	VSNL1 expression correlated with miR-124 dynamics	[69]
	Ku70	MCAO, SDR	After 2 h of focal cerebral ischemia and 24 h reperfusion, miR-124 was downregulated and Ku70 upregulated. Intraventricular administration of miR-124 antagomir prevented neuronal cells death and decreased infarct size	[89]
	JAG1	MCAO, Wistar, SVZ NC	miR-124 levels are decreased in subventricular zone (SVZ) after MCAO and via targeting JAG-1, miR-124 affects Notch signalling pathway and reduces progenitor cells proliferation and promotes their differentiation	[90]
	Usp14	MCAO, C57BL6/N, CN	Lentiviral induced expression of miR-124 had protective effect on cortical neurons when OGD was applied and similarly, miR-124 application in animal model led to decreased infarct size and smaller functional impairment. This is mediated via miR-124-Usp14-REST pathway	[91]
	Bcl-2, Bcl-xl	MCAO, C57BL/6, SHR, CN	miR-124 levels are increased after MCAO in animal model and injection of agomir for miR-124 reduces infarct size. Pro/anti-apoptotic signalling via Bcl genes is involved in observed effect	[92]
	iASPP	MCAO, C57BL/6, N2A cells	miR-124 was increased after cerebral ischemia	[93]
miR-145	SOD2	MCAO, SHR	miR-145 is increased after transient focal ischemia, targets SOD2 and application of antagomir against miR-145 increases SOD2, however, without any significant neuroprotection	[67]

(continued)

Table 9.1 (continued)

miR	Target	Model	Function in stroke	References
miR-181	HspA5 (GRP78)	MCAO, CB57/B6, astrocytes	miR-181a is upregulated in ischemic core and downregulated in surrounding penumbra. Increase in miR-181a promotes neuronal cell death due to decrease in GRP78	[94]
	Bcl-2, Mcl-1	MCAO, SDR, N2A cells, astrocytes	Decreased miR-181a after OGD reduces apoptosis and mitochondrial dysfunction in cultured astrocytes and N2A cells. In animal model of MCAO, application of miR-181a prior to ischemic insult resulted in reduced infarcted area and prevented loss of CA1 pyramidal neurons	[95, 96]
	Bcl-2, XIAP	MCAO, CB57/B6	miR-181a antagomir treatment after stroke reduced infarction size, inflammatory response (NFκB activation, leukocyte infiltration) and improved neurological deficits in mice	[97]
miR-210	VEGF pathway	MCAO, C57BL/6	Using lentiviral vector, miR-210 was introduced to experimental animals and this caused increase in new microvessels and increase in neural progenitor cells in SVZ	[98]
	Notch pathway	MCAO SDR, HUVEC	Increase in miR-210 (using lentiviral vector) caused increased angiogenesis and thus recovery after MCAO	[99]
miR-223	AQP4	MCAO SDR	Increased expression of miR-223 correlated with decreased expression of aquaporin 4	[69]
	GluR2, NR2B	miR-223 KO mice, hippocampal NC culture	miR-223, via targeting glutamate receptors (AMPAR and NMDAR) subunits (GluR2 and NR2B, respectively) prevents glutamate-induced neurotoxicity after stroke	[100]

MCAO middle cerebral artery occlusion, *OGD* oxygen-glucose deprivation, *HEK* human embryonic kidney cells, *NC* neural cells, *NBLC* neuroblastoma cells, *CN* (primary) cortical neurons, *SDR* Sprague-Dawley Rats, *SHR* spontaneously hypertensive rats, *IGF* insulin growth factor, *Bcl-2* B-cell lymphoma 2, *DNMT3a* DNA methyltransferase 3A, *Bcl2L2* Bcl-2-like protein 2, *Birc2* baculoviral IAP repeat containing 2, *Bak1* BCL2-antagonist/killer 1, *VSNL1* visinin like 1 protein, *JAG1* jagged 1, *Usp14* Ubiquitin carboxyl-terminal hydrolase 14, *iASPP* inhibitor of apoptosis-stimulating protein of p53, *SOD2* superoxide dismutase 2, *HspA5* heat shock protein A5, *Mcl-1* myeloid cell leukaemia 1, *XIAP* X-linked inhibitor of apoptosis protein, *VEGF* vascular endothelial growth factor, *AQP4* aquaporin 4, *GluR2* AMPA receptor subunit GluR2, *NR2B* N-methyl D-aspartate receptor subtype 2B

Table 9.2 Summary of other miRNAs involved in stroke pathogenesis

miR	Target	Model	Function in stroke	References
miR-17-92	PTEN	MCAO C57BL/6 J SVZ NC	After ischemic insult to animals or cell, miR-17-92 is increased. This upregulation leads to increase in neural progenitor cells proliferation via PTEN, Wnt and c-Myc related pathways	[101]
miR-103	NCX	MCAO SDR OGD BHK, PC-12 cells, CN	Blockage of miR-103-1 with specific antagomir prevents downregulation of NCX and increases recovery and decreases infarct size in experimental animals	[102]
miR-107	GLT-1	MCAO SDR ↓O2 NG108-15 cell	Expression of miR-107 is upregulated in plasma and brain after MCAO, which downregulates GLT-1 and leads to glutamate accumulation and excitotoxicity	[103]
miR-134	HSPA12	MCAO C57BL/6 J, OGD CN	Downregulation of miR-134 reduces neuronal cell death via increase in protective heat shock protein HSPA12	[104, 105]
miR-137	Grin2A	MCAO SDR, HEK-293	miR-137 is downregulated in brain after stroke as well as after mild stress, a model of depression in rats. Upregulation of miR-137 levels restored behavioural changes, probably partially via targeting Grin2A, a component of NMDA receptor	[106]
miR-200c	Reelin	MCAO CB57/B6 H_2O_2 exp., N2A cells	Intracerebroventricular pretreatment with miR-200c antagomir results in decreased infarct size and neurological deficit, partly by targeting Reelin, protein known to be involved in neurodevelopment	[107]
miR-376-5p	HIF1α	MCAO, HUVEC	miR-376-5p is downregulated after stroke and it alters angiogenesis via affecting VEGF/Notch signalling in a HIF1α dependent manner	[108]
miR-424	CDK6, CDC25A CCND1	C57BL/6 J, murine BV2 microglial cells	miR-424 is decreased after stroke. Pretreatment and post-treatment of MCAO animals with miR-424 mimic resulted in decrease in cerebral infarction and brain edema probably via affecting microglia activation and cell cycle progression	[109]
	Nrf2	MCAO, C57BL/6 J, CN	miR-424 levels increased at 1 and 4 h and decreased 24 h after ischemia in peri-infarct cortex. By affecting levels of antioxidative enzymes, miR-424 overexpression caused protection from oxidative stress which resulted in reduced neuronal cell death	[110]

MCAO middle cerebral artery occlusion, *OGD* oxygen-glucose deprivation, *HEK* human embryonic kidney cells, *NC* neural cells, *NBLC* neuroblastoma cells, *CN* (primary) cortical neurons, *SDR* Sprague-Dawley Rats, *SHR* spontaneously hypertensive rats, *PTEN* phosphatase and tensin homolog, *NCX* sodium-calcium exchanger, *GLT-1* Glial Glutamate Transporter 1, *HSPA12* heat shock protein A12, *Grin2A* glutamate receptor subunit epsilon-1, *HIF1α* Hypoxia-inducible factors 1α, *CDK6* cyclin dependent kinase 6, *CDC25A* cell division cycle 25A, *CCND1* cyclin D1, *Nrf2* nuclear factor erythroid 2-related factor

represents one of the most abundant miRNA families in the brain, where it fulfils crucial roles in neurogenesis and differentiation, as recently reviewed [80].

In the pathophysiology of stroke, let-7 may be involved in neurogenesis [111], angiogenesis and inflammation. It is one of the miRNAs transferred via exosomes affecting the gene expression of recipient cells in the atherosclerosis process [112, 113]. Expression of let-7 family members was repeatedly shown to be downregulated in above-mentioned profiling studies [68, 69, 71], and specific blockade of let-7f using antagomir led to increased neuroprotection in the rat model via affecting the insulin like growth factor (IGF-1) signalling pathway [81]. It can be hypothesized that a downregulation of let-7 family members after the stroke leads to activation of neuroprotective mechanisms mediated at least partly by the IGF-1 signalling.

miR-21

In animal brains, miR-21expression has been reported in profiling studies as increased [69, 77] and being even more dominantly increased in small artery stroke patients [77]. miR-21 is generally known to be involved in ischemia and hypoxia related processes and also has a strong antiapoptotic effect [114].

In the study by Buller and colleagues, expression of miR-21 has been observed to be significantly increased in ischemic boundary zone as compared to contralateral neurons in the MCAO animal model. However, in OGD cultured cortical neurons, no such upregulation of miR-21 has occurred [82]. On the other hand, when miR-21 was introduced to the OGD-cells, much better survival due to decreased apoptosis via downregulation of Fas-Ligand has been proven [82]. In accordance with this, reoxygenation of the N2A cells after OGD increased miR-21 levels and also promoted N2A cells survival [83].

Taking into account that miR-21 is known to be involved not only in cerebral, but also in myocardial ischemia, where it was shown to be produced by fibroblasts in the site of infarction having antiapoptotic effect on surrounding cardiomyocytes [115], strategies leading to increased miR-21 expression may potentially serve as novel future therapeutic strategies in ischemic stroke.

miR-29 Family

miR-29 family is represented by four members: miR-29a, -29b-1, -29b-2 and 29c. With many potential targets of all family members, there is still a controversy, whether miR-29 is pro-survival or pro-apoptotic since the upregulation of miR-29b has been recognized as a neuronal survival factor interfering with apoptosis by affecting Bcl-2 and BH3 pathways [84, 116], while the downregulation of miR-29 protects hearts against ischemia-reperfusion injury [117]. Luciferase target assays have indicated that miR-29 family members target both pro- and anti-apoptotic Bcl-2 family members, and thus it is necessary to evaluate this miRNA family functions in the context of disease and model chosen in particular studies [117].

Focusing more on stroke, both the downregulation [85, 87, 116] and upregulation [86, 88] of miR-29 family members have been observed in animal MCAO models with various interpretation of observed findings. Khanna et al. and Pandi et al. reported miR-29b and miR-29c downregulated in ischemic area. This loss of miR-29b/c participated on ischemic brain damage and can be prevented by oral administration of α-tocotrienol or by re-induction of both mentioned miRNAs in the brain—all these actions resulted in decreased volume of infarct and promoted cell survival. Conversely, recently Huang et al. reported the downregulation of miR-29c after the fastigial nucleus stimulation, which is known to be neuroprotective and indeed, overexpression of miR-29c resulted in increased infarct volume and poor neurological outcomes [87]. In accordance with Huang et al., results of Shi et al. showed that miR-29b was upregulated after ischemic event and promoted neuronal cell death by targeting Bcl2L2 [86].

Slightly different approach was chosen by Dhiraj et al. [88], whose group decided to study miRNA expression in salvageable tissue called penumbra. After the three vessel occlusion followed by 24 h of reperfusion, unilateral hemispheric lesion was induced and comparison of miRNAs expressed in brain ischemic cells and cultivated neuronal cells has been performed. The miR-29b-2, miR-19b, miR-339-5p and miR-341 were found to be dysregulated in both models of ischemic damage suggesting their possible participation in the regulation of gene expression in salvageable ischemic penumbra. The authors suggested also above-mentioned Bcl-2 targeting as one of potential mechanisms [88].

miR-124

miR-124 is almost exclusivelyexpressed in the central nervous system and participates in the regulation of neuronal differentiation and adult neurogenesis [118], which makes this miRNA to be referred as "brain-specific miRNA" [119]. Already in 2009 Laterza et al. showed that brain injury (induced by MCAO) caused miR-124 upregulation in plasma, similarly to elevation of miR-122 after liver injury and miR-133 after muscle injury [119] and suggested this miRNA as possible tissue-specific biomarker. Similarly, Weng et al. using the expression profiling of 671 miRNAs in various tissues (brain, heart, lung, liver, kidney, colon, skin, skeletal muscle and pancreas) confirmed miR-124 to be preferentially expressed in the nervous system, especially in the cerebellum, cerebrum and occipital and temporal lobes, and to be present in serum 6 and 48 h after the MCAO in rats [72]. Later on, elevation of circulating miR-124 was also confirmed in the profiling study by Jeyaseelan and colleagues [69]. Moreover, they reported the visinin like 1 protein (VSNL1), a neuronal calcium sensor abundantly expressed in the central nervous system as the miR-124 target in animal stroke model [69]. Nowadays, VSNL1 is a protein studied as a potent biomarker of various dementias [120] and also miR-124 is supposed to be involved in pathophysiology of dementia by targeting β-site APP cleaving enzyme 1 (BACE1) [121]. Studies of post-stroke related neuronal damage, post-stroke cognitive decline and dementia development should definitely focus on this possible interconnection. Concerning miR-124, other numerous functions within

the brain tissue, several other targets and pathways have already been identified and are summarized in Table 9.1.

Interestingly, results concerning the miR-124 up- or downregulation after ischemic insult are contradictory, which is to our opinion caused by differences among individual studies concerning sample size, MCAO induction technique, sample collection, RNA isolation, choice of internal control and other aspects.

There are two studies reporting miR-124 downregulation after stroke in animal model of MCAO. Zhu et al. reported Ku70 (DNA repair protein), upregulation after MCAO and this upregulation may further be induced via the antagomir-124 application prior to the MCAO resulting in decreased volume of infarction [89]. A slightly different study was performed by Liu and colleagues, where the authors reported miR-124 downregulation 7 days after the MCAO. This was correlated with neuronal progenitor cells proliferation. Artificial administration of miR-124 caused decrease in progenitor cells proliferation and increase in their differentiation, thus also promoting global damage to the brain [90]. Thus, both of these studies have suggested miR-124 downregulation as a protective mechanism for brain tissue repair after stroke.

However, in two other studies, similar properties of miR-124 upregulation, i.e. preventing tissue-injury, were described [91, 92]. In the first study mentioned, miR-124 upregulation after both OGD and MCAO in cell-line and animals, respectively, were confirmed and this prevented neuronal apoptosis. Authors revealed Ups14, (de)ubiquitination-related protein, as a target of miR-124 and revealed that via Ups14 targeting, miR-124 indirectly increases levels of RE1 silencing transcription factor (REST), which upregulation was reported in neurons destined to death [91]. Thus, miR-124 application led to decreased infarct volume size and also to decreased functional impairment, determined by the Rotarod test, tight rope test and modified Morris maze test. In the latter study, miR-124 upregulation in ischemic penumbra was reported and functional studies using miR-124 knockdown/increase led to infarct volume size increase/reduction, respectively. Similarly, in the primary cortical neurons, OGD caused miR-124 upregulation and miR-124 agomir and antagomir delivery reduced or promoted cell death. These effects of miR-124 are probably mediated via Bcl-2 and Bcl-xl pathways [92].

Most recently Liu and colleagues elegantly demonstrated that miR-124 upregulation in infarcted area caused the downregulation of inhibitory member of the apoptosis-stimulating proteins of p53 family (iASPP), and thus affecting the p53-mediated neuronal cell death [93]. Compared to the aforementioned studies by Doeppner and colleagues and Sun and colleagues, who reported upregulation of miR-124, this is not beneficial for neuronal cells—blockage of miR-124 with specific antagomir caused upregulation of iASPP and this resulted in reduced infarct size and reduced neuronal cell death.

It can be concluded that miR-124 is brain-specific microRNA with plentiful roles in the regulation of neuronal cell death, apoptosis and proliferation. Since the results of so far conducted studies are contradictory, more studies are needed to confirm the exact roles of miR-124.

miR-145

miR-145 should be briefly mentioned also in the stroke field, even though it is much more connected with the vessel biology. miR-143/145 cluster is involved in the function of ECs and VSMCs; however, its levels have been reported to be upregulated in brain tissue after ischemic insult [67]. Using bioinformatics prediction, superoxide dismutase 2 (SOD2) was revealed as miR-145 target and application of antagomir against miR-145 led to SOD2 increase, however, without any significant increase in the neuroprotection [67]. Recently, miR-145 was shown to be connected to microglia function and being identified as one of the p53 targets during ROS exposition [122]. Since oxidative stress is connected with reperfusion injury, potential roles for miR-145 in oxidative stress control, exceeding its known roles in vessel biology, may come up in the future.

miR-181 Family

miR-181 family represents a highly conserved group of miRNAs that consists of four members named consecutively miR-181a, -181b, -181c and 181d, and that is highly expressed in mammalian brain [123]. Main roles of this miRNA family was reported in the hematopoiesis and inflammation [124]; however, from the molecular point of view, miR-181 family targets multiple mRNAs of proteins involved in apoptosis (e.g. Bcl-2 family of antiapoptotic proteins [125]) or in tissue protection (e.g. protective proteins of endoplasmatic reticulum or heat shock proteins [94, 126]). Ouyang and colleagues focused repeatedly on various aspects of miR-181 functions in the context of cerebral ischemia concerning regulation of oxidative stress or functions of molecular chaperons [126, 127]. In their very first study, they identified miR-181a to be upregulated in ischemic core and conversely downregulated in the surrounding penumbra corresponding with the potential of the cells to die in core and survive in penumbra [94]. Accordingly, supplementation or inhibition of miR-181 in cultured astrocytes increased and reduced astrocytes cell death, respectively, similarly to effects observed in vivo. One of the targets found to be responsible for observed effect was GRP78, one of the well-known heat shock proteins—if GRP78 levels were maintained stable, miR-181a administration did not promote the cell death [94]. Concerning further delayed neuronal cell death, miR-181 was shown to indirectly regulate the glutamate transporter (GLT-1), which induction is known to prevent the brain from excitotoxicity induced by neurotransmitter glutamate [128].

In agreement with the previous studies, application of miR-181a mimics prior [95] or after [97] the ischemic insult resulted in neuroprotection. Especially the study by Xu and collaborators has shown a great potential of miR-181 application. Since in the clinical practice, clinicians are typically faced with patients after the onset of stroke with no possibility to pretreat them. Xu and colleagues applied miR-181 antagomirs either intravenously (1 h after the MCAO) or intracerebroventricularly

(2 h after the MCAO) and interestingly both ways of application have shown reduced infarction volume (at about one third at 48 h after reperfusion). Neurologic deficit was assessed by the Rotarod test and was significantly improved as well as the overall 4-weeks survival (90 % versus 72 % in sham) and neurobehavioural recovery. In conclusion, miR-181 antagomir treatment showed great promises for the future therapeutic applications in the early post-stroke treatment.

miR-210

miR-210, a master hypoxia-miR, represents a hypoxia-induced and anti-apoptotic miRNA with approximately 35 specific transcripts as its direct or indirect targets [129]. Its overexpression in normoxic human endothelial cells stimulates formation of capillary-like structures, and increases cells migration and differentiation [130]. Consistent with the reported induction under hypoxic conditions via HIF-dependent and independent pathways [129], miR-210 expression was reported to be higher in ischemic brain, where it plays many protective roles against ischemia induced injury [98, 99]. In the setting of cerebral ischemia in rat or mice, increase in miR-210 by lentiviral vector led to increased number of new vessels [99] and progenitor cells in subventricular zone [98], thus resulting in increased angio- and neurogenesis, and preventing mice from ischemia-induced injury. To sum up miR-210 represents another promising target for stroke therapeutics.

miR-223

From the more deeply studied miRNAs, as the last one miR-223 will be mentioned. Together with miR-130a and miR-320, this miRNA was reported to target aquaporin genes, especially aquaporin 4 that is typically expressed in the brain [131]. miR-320a was shown to directly affect the expression of aquaporin 4 and aquaporin 1. Blockade of miR-320a with antagomir led to increase in the aquaporin expression and reduction of infarct volume [131]. Similar results were obtained with the use of antagomir-130a. Reduction in infarct size and increased neuronal recovery were observed after the application [132].

miR-223 is of further interest, since except of its proved aquaporin targeting [69], it was also reported as a platelet-secreted [133] and lipoprotein particle-transferred [134] miRNA, playing one of the central roles in the cholesterol metabolism [135]. Similarly to miR-181 mentioned above and miR-107 mentioned further, miR-223 also has a potential to protect brain from glutamate excitotoxicity by targeting the glutamate receptor subunits GluR2 and NR2B [100]. Moreover, in the study by Duan et al. it was observed that miR-223 levels were lower in patients with diabetes mellitus, which promoted platelet activation and increased the risk of stroke development in DM patients [136].

Other miRNAs in Ischemic Stroke

From the profiling studies mentioned at the beginning of the chapter it is obvious, that more miRNAs are involved in the pathophysiology of acute stroke than those described above. However, information regarding their function still need to be determined or is of a limited extent, thus we provide only basic information regarding their pathophysiologic, diagnostic or therapeutic potentials.

Due to its involvement in general cellular processes, miR-17-92 cluster has been described to be involved in the pathophysiology of almost any disease [137], including stroke [101]. In the subventricular zone of animals after MCAO, miR-17-92 cluster levels were increased and this, via targeting of PTEN, increased a proliferative capacity of neural progenitor cells, as confirmed also in vitro.

Following the numerical order, other miRNA involved in stroke is miR-103. It is responsible for a regulation of Na^{+}–Ca^{2+} exchanger (NCX). Its downregulation by a specific antagomir reduced brain damage and neurological deficit in experimental animals [102]. Like above-mentioned miR-181 family, also other miRNAs were shown to affect the glutamate signalling (particularly miR-107 [103]) or target heat-shock proteins (particularly HSPA12), e.g. miR-134 [104, 105]. Another miRNA recently described to promote stroke damage is miR-200c. Its blockade increased levels of Reelin, thus promoting neuronal migration and synaptogenesis, resulting in neuroprotection [107]. Concerning angiogenesis, similar effects to miR-210 were observed in miR-376-5p. This miRNA affects the VEGF/Notch signalling modulating angiogenesis as well [108].

Last but not the least, miR-424 was shown to be downregulated in brain tissue within the profiling studies [69] and it was also shown to be downregulated in peripheral leukocytes in patients after stroke [109, 110]. Restoration of miR-424 activity led to decrease in infarct volume and neuronal cell death, which was associated with decreased microglia activation [109] and reduced oxidative stress [110].

Candidate microRNAs as Biomarkers of Acute Stroke

Using animal models, elevation of serum miR-124 was reported in two independent studies and this upregulation was consistent for at least 48 h after stroke induction [72, 119], which would make miR-124 as a potential stroke biomarker. However, no correlations with outcome of experimental animals or infarct size were observed [72, 119]. Interestingly, in human studies, levels of miR-124 were reported both to be upregulated [138] and downregulated [139] after acute stroke. In the study by Leung et al., upregulation of miR-124 was prominent only in the early phase (below 6 h). In the further sampling, levels of miR-124 were significantly lower compared to acute phase [138]. Leung et al. moreover showed that miR-124 levels were higher in patients with haemorrhagic stroke compared to ischemic stroke only [138]. In another study by Liu et al., miR-124 levels were downregulated and they negatively correlated with infarct volume, CRP levels and levels of matrix metalloproteinase 9

(MMP9), thus somehow reflecting damage of neural tissue and inflammatory activity. However, no correlation with stroke severity defined by NIHSS was observed [139]. Similar properties were observed also for another neuri-miR, miR-9. The true diagnostic potential of miR-124, although being termed as brain specific, needs to be determined in larger multicentric studies since the results of currently conducted studies are contradictory.

Leaving miR-124 and moving further with human studies, let-7, miR-21, miR-30a and miR-126, miR-221 levels were studied as potential diagnostic and prognostic biomarkers.

Concerning let-7, miR-30a and miR-126, altogether 247 patients at different time points after stroke (in the acute phase within 24 h, in the subacute phase within 1 week and in the chronic phases within 48 weeks after stroke) were enrolled. It was observed that let-7 levels were decreased after stroke with a slow restitution after the 24th week. Interestingly, levels of let-7 were more profoundly decreased in patients with stroke of atherosclerotic aetiology [140]. Cut-off value for the let-7 was calculated to 1.675 at 24 h with the sensitivity 92 % and specificity 84 %.

Another larger study was performed in 233 patients (partly patients with ischemic stroke and partly patients with carotid atherosclerosis without ischemic stroke) and 157 controls focusing on miR-21, miR-221 and miR-145 levels in the serum after ischemic stroke [50]. miR-21 and miR-221 showed opposite trends, i.e. miR-21 being upregulated and miR-221 being downregulated in patients compared to the controls. Concerning miR-145, its levels were not detected in more than 50 % of study subjects and its potential for stroke diagnosis is thus not very promising [50]. However, if the whole blood RNA was isolated, miR-145 levels were shown to be upregulated after ischemic stroke in a smaller study by Gan et al. [49] and also in the profiling study performed by Sepramaniam et al. [74]. Gan et al. moreover suggested increase in miR-145 to be predictive for better outcome, due to miR-145 targeting of KLF-4/5 and its effect on reendothelization [49].

Promising results were observed for miR-210, previously mentioned as hypoxia-miR, which levels were reported to be downregulated in peripheral blood leukocytes in patients after stroke. Decrease in miR-210 levels in these patients was continuous and deepened within 2 weeks from stroke onset [141]. Moreover, after the correlation with NIHSS and mRS, patients with higher miR-210 levels had better prognosis, compared to patients with the lowest miR-210 expression. Zeng et al. further determined miR-210 peripheral blood mononuclear cells (PBMC) levels together with the levels of other potential biomarkers, including various cytokines (e.g. IFNγ, IL-1, IL-6, TNFα and others) and haemostatic markers (fibrin and its degradation products FDP, D-dimers, etc.). Combination of miR-210, FDP and IL-6 had even higher sensitivity and specificity for stroke outcome prediction (defined by mRS after 3 months) compared to the use of individual markers alone [142].

Last but not the least, also miR-223 was suggested as a potential biomarker of acute stroke [143]. After dissociation of erythrocytes, RNA was isolated from the remaining cells and miR-223 levels were determined to be increased after stroke with more prominent increase in large and small vessel stroke subtypes. Moreover, a negative correlation with NIHSS (and in animal part of the study with infarct size)

and a positive correlation with circulating IGF-1, one of miR-223 targets, were observed. Due to its pleiotropic effect, miR-223 specificity for stroke needs to be tested in other consecutive studies.

Recently, Kim et al. focused on atherosclerosis related miRNAs in the diagnostics of acute stroke. They revealed that miR-126 levels correlated with brain atherosclerotic damage and that levels of miR-17 were increased after acute stroke being also predictive of early stroke recurrence (within the study period, thus probably 1 year—only abstract was available to authors during the manuscript preparation [66]).

Future Directions

In conclusion we would like to point out the tremendous development and progress that has been done regarding the roles of miRNAs in ischemic stroke. Studies above are definitely informative about the involvement of miRNAs in the stroke pathophysiology and also about their potential clinical utilities as diagnostic, prognostic and even therapeutic tools.

For future research, it is needed to define the standardized protocols of miRNAs detection, extraction and isolation. Current studies differ and vary a lot in the source of miRNAs—in animal studies, sometimes ischemic core and sometimes penumbra have been used and this has not been quite often precisely specified; in the human studies, miRNA source ranged from the whole blood through plasma or sera to the isolated cells only. Also, various internal controls are used, sometimes using additive cel-miR-39, sometimes using miR-16 or another defined ubiquitous miRNA. Universal internal control should soon be established to normalize the data from various studies and obtain clearer and more normalized results. Also the need of multicentric studies employing more than 300 subjects is anticipated and this inquires specific needs of proper and universal sample storage protocols.

Last but not the least, the miRNA potential in stroke therapy should be further investigated and focused not only on effects of injected ago- or antagomiRs, but also determining potential side effects, mechanisms of uptake and elimination. Also, the ways of application should be precisely defined in order to increase specific targeting of brain tissue.

Intracranial Aneurysms

Epidemiology of Intracranial Aneurysms

Intracranial aneurysms represent acquired lesions of cerebral arterial wall occurring in 0.5–3 % of general population and accounting for 80–85 % of non-traumatic subarachnoid haemorrhages (i.e. bleeding into subarachnoid space).

These saccular-shaped lesions are typically localized in proximal arterial bifurcations. Typical anatomical localizations are as follows: internal carotid artery (ICA), anterior communicating artery (ACom), middle cerebral artery (MCA), tip of basilar artery (BA) and arteries of the cerebellum.

Risk factors associated with higher occurrence include higher age, female sex, cigarette smoking, hypertension and genetic predisposition (e.g. autosomal dominant polycystic kidney disease, Marfan's syndrome, Ehlers-Danlos syndrome type IV) [144–147].

Clinicians are faced with a decision regarding an optimal management of intracranial aneurysms; however, there are still many unsolved controversies regarding the risk of rupture and treatment of smaller asymptomatic aneurysms due to lack of randomized control trials. Decisions are thus made regarding patient history data, size and location of aneurysm/s, expert opinion and guidelines.

On the one hand, the particular effect of aneurysms growth on subsequent risk of rupture is not determined; on the other hand, patients with obvious enlargement should be strongly considered for interventional treatment (endovascular coiling or neurosurgical clipping, Fig. 9.3 [148]).

For those treated conservatively, proper management of hypertension, smoking cessation and repeat CT angiography or MR angiography are strongly recommended (Fig. 9.4) [144].

Biomarkers predicting the clinical course of intracranial aneurysm are still lacking. As long as miRNAs represent potential biomarkers sensitively reflecting the diseases state, maybe their potential role in the monitoring of aneurysms will come up in the near future.

microRNA Profiling Studies and Aneurysms

Animal Studies

Up to date there is only one study using animal model performed by Lee et al. in 2013 [149]. Aneurysms formation was induced by ligation of the left common carotid artery and the posterior branches of both renal arteries in Sprague-Dawley rats. Biological sampling from aneurysms (dissected out of left posterior communicating artery) or from healthy arteries (dissected out of the circles of Willis) were obtained and subsequent miRNA array profiling was done identifying 14 miRNAs upregulated (miR-1, miR-21, miR-22-5p, miR-24-1-5p, miR-26b, miR-29a, miR-29b, miR-29c, miR-101b, miR-140, miR-147, miR-181c, miR-223, miR-451) and 6 miRNAs downregulated (miR-92b, miR-138, miR-181d, miR-433, miR-489, miR-551b) in aneurysmatic vessels. Detected miRNAs are known to be involved in inflammation, apoptosis or angiogenesis and more detailed studies are definitely needed to validate the profiling results and to better understand the intracranial aneurysms development.

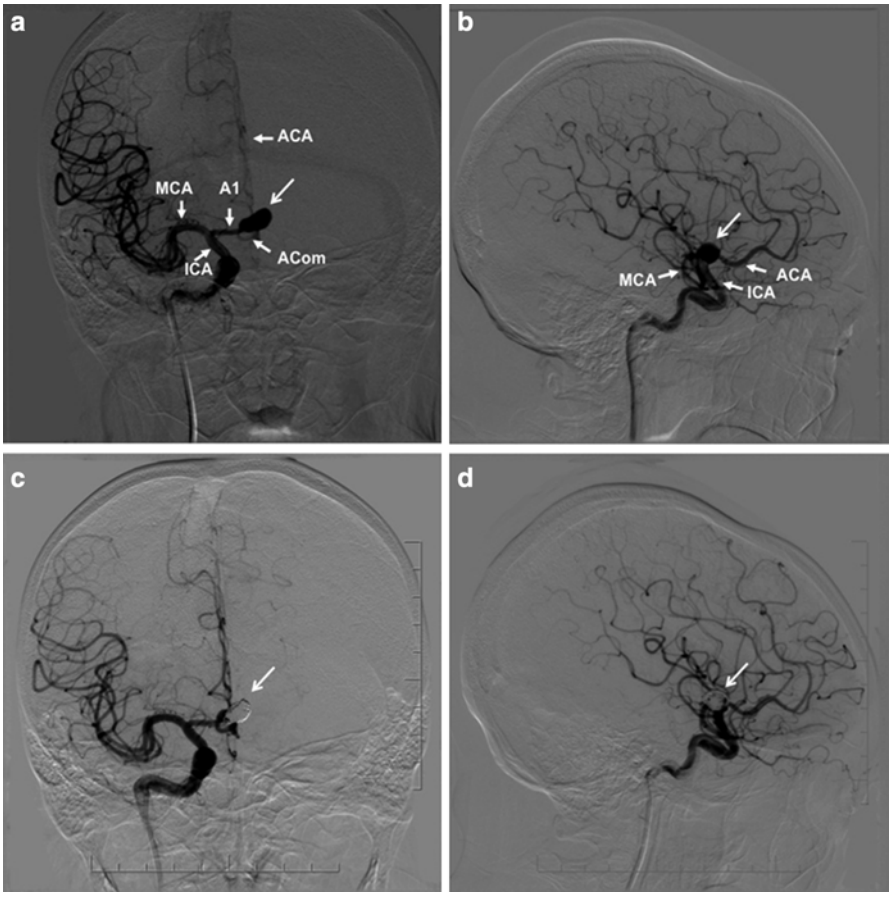

Fig. 9.3 Endovascular coiling of ruptured anterior communicating artery aneurysm, digital subtraction angiography images (DSA images). Seventy-five-year-old female with a ruptured aneurysm as a source of subarachnoid haemorrhage indicated for interventional treatment. (*top*) Subtracted right internal carotid artery angiograms in antero-posterior (*top left*) and lateral (*top right*) view showing aneurysm (*open arrow*) of the anterior communicating artery of size 12×6 mm (*bottom*). Final control series after the endovascular coiling showing almost total obliteration of the aneurysmal sac. *A1* A1 segment of the anterior cerebral artery, *ACA* A2 and A3 segments of the anterior cerebral artery, *ACom* anterior communicating artery, *ICA* internal carotid artery, *MCA* middle cerebral artery

Human Studies

Unlike in the case of stroke in human studies biological material other than bodily fluids can be collected when the intracranial aneurysms (IAs) are surgically treated. So far, there were two profiling studies using tissue obtained from patients during the surgery and two studies focusing on detection of intracranial aneurysms using circulating microRNAs.

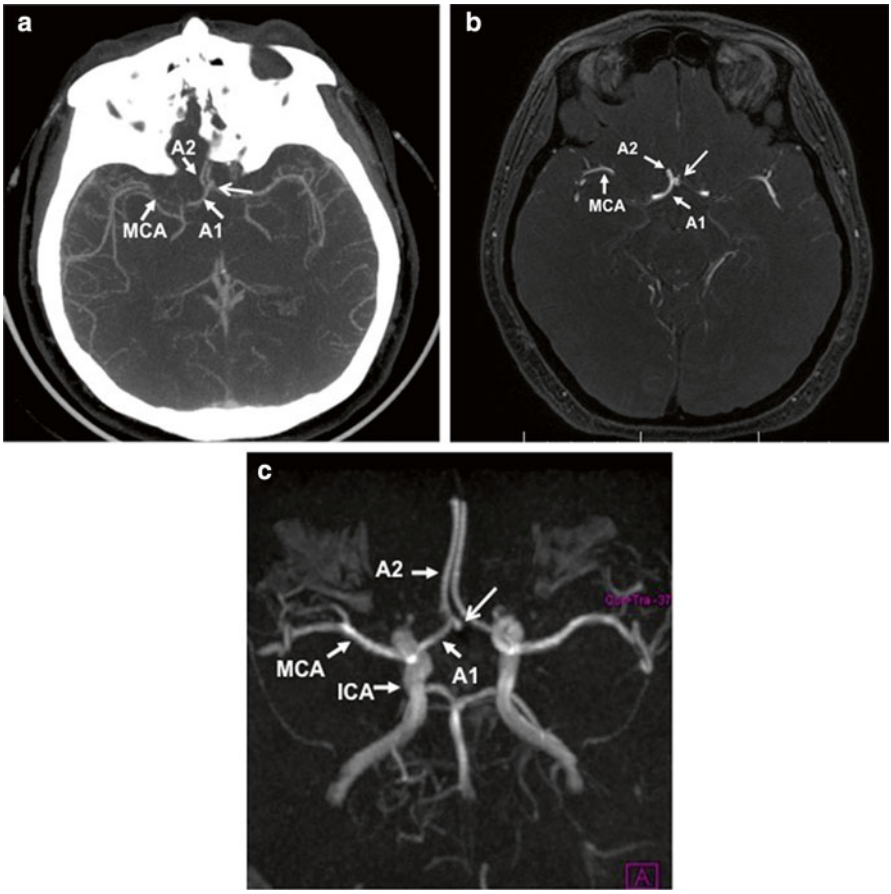

Fig. 9.4 Management of residual aneurysm sac after endovascular coiling. Forty-two-year-old female with anterior communicating artery aneurysm indicated for endovascular coiling. A tiny residual aneurysm was revealed on MRI control scanning 6 months after the procedure (conservative approach with regular MRI controls was recommended). (*top left*) CT angiography showing the aneurysm (*open arrow*) of the right anterior communicating artery (*top right*) MR angiography revealing a residual filling of the aneurysm (*open arrow*), size of 2 mm (*bottom*) MRA angiography reconstruction of the circle of Willis. *A1* A1 segment of the anterior cerebral artery, *A2* A2 segment of the anterior cerebral artery, *ICA* internal carotid artery, *MCA* middle cerebral artery

In the study by Jiang et al. (2013) microarray analysing of full-thickness vessel wall samples from 14 ruptured IA patients and 14 controls was made. Thirty differentially expressed miRNAs were found to be dysregulated and 18 miRNAs were found to be significantly different between IAs and control group (miR-1, miR-7-1-3p, miR-23b-3p, miR-24-1-5p, miR-28-5p, miR-28-3p, miR-29b-2-5p, miR-29c-5p, miR-29c-3p, miR-133a, miR-133b, miR-140-3p, miR-143-5p, miR-143-3p, miR-145-5p, miR-145-3p, miR-455-5p) [150]. One year later, Liu et al.

screened IA samples and found 157 differentially expressed (72 upregulated and 85 downregulated) miRNAs. Out of these, let-7a, miR-1, miR-30c and miR-101 were shown to be involved in the regulation of programmed cell death, extracellular matrix organization response to oxidative stress, TGF-β signalling pathway and smooth muscle proliferation [151].

Profiling studies focusing on circulating miRNAs were performed by Jin et al. in 2013 [152] and Lie et al. in 2014 [153]. In the study by Jin et al., blood samples of 24 IA patients were analysed. Patients were divided into four groups according to aneurysm characteristics (including aneurysms with/without daughter aneurysm, ruptured aneurysms and angiography-negative group). Aneurysms were located in the ophthalmic artery, basilar artery, posterior communicating artery and internal carotid artery. Altogether 86 miRNAs (69 upregulated, 17 downregulated) were identified by microarray study. In the larger study by Li et al., authors included 40 IA patients (20 with ruptured IA and 20 with unruptured IA) and 20 healthy volunteers (having neurological symptoms, such as headache, indicated for digital subtraction angiography) [153]. One hundred nineteen miRNAs were significantly changed in patients with unruptured aneurysms and 23 miRNAs in patients with ruptured aneurysms (20 of them in both ruptured and unruptured patients). miR-16 and miR-25 were studied in more detail, because these miRNAs were the most abundant in plasma. Using logistic regression, authors proved hypertension and levels of these two miRNAs, as independent predictors for the presence of IAs.

Candidate miRNA Studies and Intracranial Aneurysms

miR-21

miR-21, as described in previous chapters dealing with stroke, has very important roles in hypoxia, apoptosis and tumorigenesis. Role in the aneurysm formation was suggested in 2012 in the study by Maegdefessel et al.—authors showed increased miR-21 expression in the developed abdominal aortic aneurysm and this upregulation seemed to be protective since miR-21 blockade caused aneurysm enlargement [154]. Shortly after this pioneering attempt, potential roles of miR-21 were also shown in IAs in profiling studies observing also increase in miR-21 levels suggesting a potential regulatory role for miR-21 in controlling aneurysmal growth [153, 154].

miR-26

miR-26 is known to be involved in programmed cell death and response to oxidative stress, TGF-β signalling pathway and importantly in smooth muscle cell proliferation—it is one of the miRNAs involved in the regulation of phenotypic switch of VSMCs through Smad1 and Smad4 proteins [155]. Within the rat model of abdominal aortic aneurysm, levels of miR-26 were found to be decreased, suggesting a

potential failure in the regulatory mechanism, since inhibition of miR-26 expression should promote apoptosis and differentiation [155]. On the contrary, in profiling studies both Lee et al. and Li et al. observed increased level of this miRNA in IA rats and patients, respectively [149, 153]. Distinct roles of miR-26 in aneurysm formation thus still needs to be clarified.

miR-29 Family

All three members of miR-29 family (miR-29a, miR-29b and miR-29c, see above) have been associated with aneurysm formation, as shown mostly on abdominal aortic aneurysms—miR-29 inhibition attenuated and miR-29 overexpression promoted abdominal aneurysm formation in various animal models [156–158]. Concerning IAs, contradictory results are currently present. While two profiling studies revealed that levels of miR-29b are robustly downregulated in IA [150, 151], other two indicated miR-29 upregulation [149, 153]. Since miR-29 is known to be involved in the formation of extracellular matrix and fibrotic processes, its potential involvement in aneurysm formation should be definitely studied into more detail [159].

miR-143 and miR-145

Considering miR-143/145 function in the VSMCs, it is not surprising that it has been studied in the field of intracranial aneurysms. In the profiling performed by Jiang et al., miR-143/145 levels were found to be decreased [150]. Elia et al. further focused on miR-143/145 functions in the development of IAs and also detected downregulation of miR-143/145 levels [36]. Downregulation of this cluster is known to promote VSMCs phenotypic switch to "proliferative" phenotype, which may theoretically results in aneurysm formation, however, since the whole research discovering the roles of miRNAs in IAs is at the beginning, more functional studies are still needed to describe precise mechanism leading to IA formation.

Brain Arteriovenous Malformations

Epidemiology of Brain Arteriovenous Malformation

Brain arteriovenous malformations (AVM) represent a complex (nidus) of abnormal arteries and veins that directly fistualize without an intervening capillary bed (Figs. 9.5 and 9.6). They differ in morphology, size, location, particular clinical symptoms and risk of rupture (and subsequent intracranial bleeding). Narrow incidence is estimated to 1.12–1.42 cases per 100,000 person years.

The first-ever haemorrhage at presentation range from 38 to 68 %. This clinical symptom represents the most common one followed by seizures and/or headache. Nevertheless, the AVMs natural history and risk of rupture is still largely unknown [160–163].

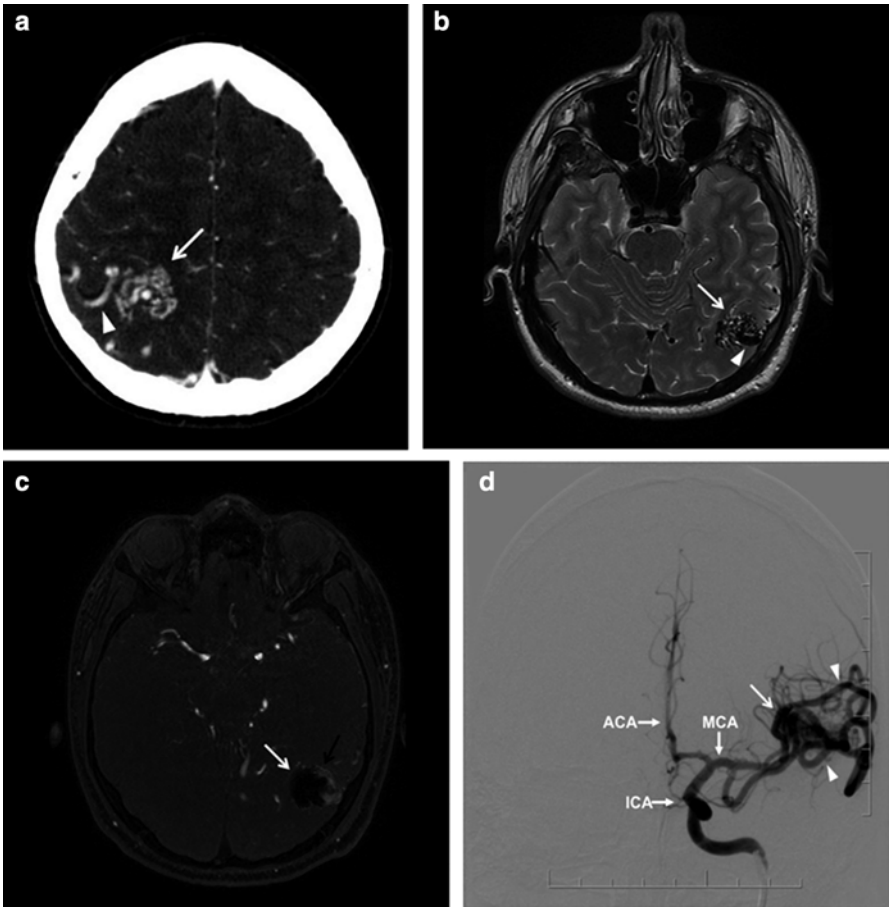

Fig. 9.5 Diagnostic imaging of brain arteriovenous malformations. (*top left*) CT angiography, scan showing a hyperdense mass of enlarged feeding arteries (*arrow head*) supplying the nidus (*open white arrow*) (*top right*) MRI, T2 weight image. The nidus appears on the scan as a hypointense mass with a feeding artery (*bottom left*) MR angiography, scan demonstrating a residual filling (*open black arrow* pointing to the hyperintense area) of the nidus after endovascular embolization of AVM (hypointense region marked with *white open arrow*) (*bottom right*) Digital subtraction angiogram demonstrates a blood supply (feeding arteries) of the AVM (*open white arrow*) from branches of the middle cerebral artery (*arrowheads*)

AVM rupture and subsequent intraparenchymal bleeding account for the most devastating complications with overall annual rate of haemorrhage for non-treated malformations 2.1–4.1 %. Based on several multivariate analyses, the nidus size (large nidus is more predictive of increased risk for future bleeding and re-bleeding), deep brain location and deep vein drainage were determined to be significant in risk for spontaneous haemorrhage [164–166].

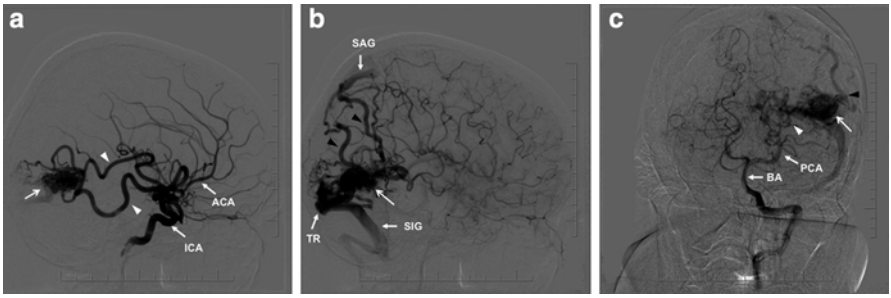

Fig. 9.6 Arteriovenous malformation on digital subtraction angiography (DSA). DSA represents a gold standard in a diagnostic management of AVM. It is widely used to delineate location, number of feeding vessels and pattern of drainage. AVM on the angiogram appears as a packed mass of enlarged feeding arteries (*white arrow heads*) that supply the central nidus (*open arrow*). Scans present a 19-year-old patient with AVM in the parieto-occipital region. The nidus is fed by vessels from carotid artery and vertebrobasilar circulation. Dilated veins (*black arrow heads*) drain the nidus into the superior sagittal sinus and transverse sinus. Abnormal venous opacification in arterial phase of DSA represents evidence for pathological shunting; (*left*) Lateral view of the AVM showing feeding arteries arising from branches of the middle cerebral artery (anterior circulation); (*middle*) Lateral view showing draining veins into the superior sagittal and transverse sinus; (*right*) Anteroposterior view showing feeding arteries arising from the posterior cerebral artery (vertebrobasilar circulation). *ACA* anterior cerebral artery, *BA* basilar artery, *ICA* internal carotid artery, *PCA* posterior cerebral artery, *TR* transverse sinus, *SAG* superior sagittal sinus, *SIG* sigmoid sinus

Studies regarding angio-architecture features (regarding muscular and elastic laminae and presence of intervening brain parenchyma), risk of rupture and AVM evolution are needed in order to help clinicians in treatment decision-making and management.

miR-18a and Cerebral AVMS

Information regarding the role of miRNAs in AVMs are limited to miR-17-92 cluster and out of this cluster almost entirely on miR-18a. As mentioned before, miR-17-92 cluster is known to play multiple functions in almost any tissue [137]. miR-18a represents an important member of miR-17-92 cluster which is involved in the regulation of angiogenesis. Antiangiogenic activity in AVM progression was particularly influenced by miR-18a (together with miR-17, miR-19a and miR-20a). Overexpression of miR-18a and miR-19a regulated the levels of thrombospondin 1 and the connective tissue growth factor (CTGF) [24, 167, 168].

Furthermore, in 2013 Ferreira et al. used AVM samples from six patients in order to determine the effect of miR-18a introduction on AVM-derived brain endothelial cells (AVM-BECs). miR-18a introduction led to increase in TSP-1 and decrease in inhibitor of DNA-binding protein 1, which resulted into better AVM-BECs function suggesting miR-18a supplementation as a potential AVM treatment [168].

Conclusions

Determining the roles of microRNAs in cerebrovascular diseases is still at its beginning; however, much progress in the field has already been done and much progress is yet to be expected. Better understanding of the post-transcriptional regulation of gene expression in endothelial cells, vascular smooth muscle cells, fibroblasts or even neurons or glia may direct further research and development of new drugs based on RNA interference for the treatment of atherosclerosis, stroke or its complications. Studies focusing on circulating miRNAs may reveal new and unexpected communication pathways among individual tissues and these findings may represent a potential source of new biomarkers, reflecting organ damage, or being predictive of the response to therapy or patients prognosis. We can definitely sum up that miRNA research hold great promises for the future therapeutics, diagnostics and for promotion of personalized medicine.

Acknowledgements J. Novak received a research support from MUNI/A/1326/2014 institutional grant of Masaryk University in Brno, Czech Republic. O. Volny received a research support from European Regional Development Fund—Project FNUSA-ICRC (No.CZ.1.05/1.1.00/02.0123). O. Volny received a student scholarship of city Ostrava in 2014/2015.

References

1. Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med. 1999;340:115–26.
2. Gallino A, Aboyans V, Diehm C, Cosentino F, Stricker H, Falk E, et al. Non-coronary atherosclerosis. Eur Heart J. 2014;35:1112–9.
3. Nam D, Ni CW, Rezvan A, Suo J, Budzyn K, Llanos A, et al. Partial carotid ligation is a model of acutely induced disturbed flow, leading to rapid endothelial dysfunction and atherosclerosis. Am J Physiol Heart Circ Physiol. 2009;297:H1535–43.
4. Inaba Y, Chen JA, Bergmann SR. Carotid plaque, compared with carotid intima-media thickness, more accurately predicts coronary artery disease events: a meta-analysis. Atherosclerosis. 2012;220:128–33.
5. Fujihara K, Suzuki H, Sato A, Ishizu T, Kodama S, Heianza Y, et al. Comparison of the Framingham risk score, UK Prospective Diabetes Study (UKPDS) Risk Engine, Japanese Atherosclerosis Longitudinal Study-Existing Cohorts Combine (JALS-ECC) and maximum carotid intima-media thickness for predicting coronary artery stenosis in patients with asymptomatic type 2 diabetes. J Atheroscler Thromb. 2014;21:799–815.
6. Rockman CB, Hoang H, Guo Y, Maldonado TS, Jacobowitz GR, Talishinskiy T, et al. The prevalence of carotid artery stenosis varies significantly by race. J Vasc Surg. 2013;57:327–37.
7. Palm F, Dos Santos M, Urbanek C, Greulich M, Zimmer K, Safer A, et al. Stroke seasonality associations with subtype, etiology and laboratory results in the Ludwigshafen Stroke Study (LuSSt). Eur J Epidemiol. 2013;28:373–81.
8. Rudd JH, Myers KS, Bansilal S, Machac J, Rafique A, Farkouh M, et al. (18) Fluorodeoxyglucose positron emission tomography imaging of atherosclerotic plaque inflammation is highly reproducible: implications for atherosclerosis therapy trials. J Am Coll Cardiol. 2007;50:892–6.

9. Truijman MT, de Rotte AA, Aaslid R, van Dijk AC, Steinbuch J, Liem MI, et al. Intraplaque hemorrhage, fibrous cap status, and microembolic signals in symptomatic patients with mild to moderate carotid artery stenosis: the Plaque at RISK study. Stroke. 2014;45:3423–6.
10. Artom N, Montecucco F, Dallegri F, Pende A. Carotid atherosclerotic plaque stenosis: the stabilizing role of statins. Eur J Clin Invest. 2014;44:1122–34.
11. Maiolino G, Rossitto G, Caielli P, Bisogni V, Rossi GP, Calo LA. The role of oxidized low-density lipoproteins in atherosclerosis: the myths and the facts. Mediators Inflamm. 2013;2013:714653.
12. Chiu JJ, Chien S. Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. Physiol Rev. 2011;91:327–87.
13. Sun X, Belkin N, Feinberg MW. Endothelial microRNAs and atherosclerosis. Curr Atheroscler Rep. 2013;15:372.
14. Loyer X, Vion AC, Tedgui A, Boulanger CM. Microvesicles as cell-cell messengers in cardiovascular diseases. Circ Res. 2014;114:345–53.
15. Kumar S, Kim CW, Simmons RD, Jo H. Role of flow-sensitive microRNAs in endothelial dysfunction and atherosclerosis: mechanosensitive athero-miRs. Arterioscler Thromb Vasc Biol. 2014;34:2206–16.
16. Treguer K, Heinrich EM, Ohtani K, Bonauer A, Dimmeler S. Role of the microRNA-17-92 cluster in the endothelial differentiation of stem cells. J Vasc Res. 2012;49:447–60.
17. Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, et al. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell. 2008;15:261–71.
18. Boettger T, Beetz N, Kostin S, Schneider J, Kruger M, Hein L, et al. Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster. J Clin Invest. 2009;119:2634–47.
19. Albinsson S, Skoura A, Yu J, DiLorenzo A, Fernandez-Hernando C, Offermanns S, et al. Smooth muscle miRNAs are critical for post-natal regulation of blood pressure and vascular function. PLoS One. 2011;6, e18869.
20. Vigorito E, Kohlhaas S, Lu D, Leyland R. miR-155: an ancient regulator of the immune system. Immunol Rev. 2013;253:146–57.
21. Wang KC, Garmire LX, Young A, Nguyen P, Trinh A, Subramaniam S, et al. Role of microRNA-23b in flow-regulation of Rb phosphorylation and endothelial cell growth. Proc Natl Acad Sci U S A. 2010;107:3234–9.
22. Fang Y, Davies PF. Site-specific microRNA-92a regulation of Kruppel-like factors 4 and 2 in atherosusceptible endothelium. Arterioscler Thromb Vasc Biol. 2012;32:979–87.
23. Kaluza D, Kroll J, Gesierich S, Manavski Y, Boeckel JN, Doebele C, et al. Histone deacetylase 9 promotes angiogenesis by targeting the antiangiogenic microRNA-17-92 cluster in endothelial cells. Arterioscler Thromb Vasc Biol. 2013;33:533–43.
24. Doebele C, Bonauer A, Fischer A, Scholz A, Reiss Y, Urbich C, et al. Members of the microRNA-17-92 cluster exhibit a cell-intrinsic antiangiogenic function in endothelial cells. Blood. 2010;115:4944–50.
25. Petrocca F, Vecchione A, Croce CM. Emerging role of miR-106b-25/miR-17-92 clusters in the control of transforming growth factor beta signaling. Cancer Res. 2008;68:8191–4.
26. Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, et al. miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell. 2008;15:272–84.
27. Liu B, Peng XC, Zheng XL, Wang J, Qin YW. MiR-126 restoration down-regulate VEGF and inhibit the growth of lung cancer cell lines in vitro and in vivo. Lung cancer. 2009;66:169–75.
28. Sessa R, Seano G, di Blasio L, Gagliardi PA, Isella C, Medico E, et al. The miR-126 regulates angiopoietin-1 signaling and vessel maturation by targeting p85beta. Biochim Biophys Acta. 1823;2012:1925–35.
29. Harris TA, Yamakuchi M, Ferlito M, Mendell JT, Lowenstein CJ. MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. Proc Natl Acad Sci U S A. 2008;105:1516–21.

30. Zernecke A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, Denecke B, et al. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci Signal. 2009;2:ra81.
31. Schober A, Nazari-Jahantigh M, Wei Y, Bidzhekov K, Gremse F, Grommes J, et al. MicroRNA-126-5p promotes endothelial proliferation and limits atherosclerosis by suppressing Dlk1. Nat Med. 2014;20:368–76.
32. Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, et al. Circulating microRNAs in patients with coronary artery disease. Circ Res. 2010;107:677–84.
33. Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res. 2010;107:810–7.
34. Zhou J, Li YS, Nguyen P, Wang KC, Weiss A, Kuo YC, et al. Regulation of vascular smooth muscle cell turnover by endothelial cell-secreted microRNA-126: role of shear stress. Circ Res. 2013;113:40–51.
35. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev. 2004;84:767–801.
36. Elia L, Quintavalle M, Zhang J, Contu R, Cossu L, Latronico MV, et al. The knockout of miR-143 and -145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease. Cell Death Differ. 2009;16:1590–8.
37. Kohlstedt K, Trouvain C, Boettger T, Shi L, Fisslthaler B, Fleming I. AMP-activated protein kinase regulates endothelial cell angiotensin-converting enzyme expression via p53 and the post-transcriptional regulation of microRNA-143/145. Circ Res. 2013;112:1150–8.
38. Hergenreider E, Heydt S, Treguer K, Boettger T, Horrevoets AJ, Zeiher AM, et al. Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. Nat Cell Biol. 2012;14:249–56.
39. Nazari-Jahantigh M, Wei Y, Noels H, Akhtar S, Zhou Z, Koenen RR, et al. MicroRNA-155 promotes atherosclerosis by repressing Bcl6 in macrophages. J Clin Invest. 2012;122:4190–202.
40. Du F, Yu F, Wang Y, Hui Y, Carnevale K, Fu M, et al. MicroRNA-155 deficiency results in decreased macrophage inflammation and attenuated atherogenesis in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2014;34:759–67.
41. Sun HX, Zeng DY, Li RT, Pang RP, Yang H, Hu YL, et al. Essential role of microRNA-155 in regulating endothelium-dependent vasorelaxation by targeting endothelial nitric oxide synthase. Hypertension. 2012;60:1407–14.
42. Tian FJ, An LN, Wang GK, Zhu JQ, Li Q, Zhang YY, et al. Elevated microRNA-155 promotes foam cell formation by targeting HBP1 in atherogenesis. Cardiovasc Res. 2014;103:100–10.
43. Wagner J, Riwanto M, Besler C, Knau A, Fichtlscherer S, Roxe T, et al. Characterization of levels and cellular transfer of circulating lipoprotein-bound microRNAs. Arterioscler Thromb Vasc Biol. 2013;33:1392–400.
44. Donners MM, Wolfs IM, Stoger LJ, van der Vorst EP, Pottgens CC, Heymans S, et al. Hematopoietic miR155 deficiency enhances atherosclerosis and decreases plaque stability in hyperlipidemic mice. PLoS One. 2012;7, e35877.
45. Ma X, Ma C, Zheng X. MicroRNA-155 in the pathogenesis of atherosclerosis: a conflicting role? Heart Lung Circ. 2013;22:811–8.
46. Cipollone F, Felicioni L, Sarzani R, Ucchino S, Spigonardo F, Mandolini C, et al. A unique microRNA signature associated with plaque instability in humans. Stroke. 2011;42:2556–63.
47. Raitoharju E, Lyytikainen LP, Levula M, Oksala N, Mennander A, Tarkka M, et al. miR-21, miR-210, miR-34a, and miR-146a/b are up-regulated in human atherosclerotic plaques in the Tampere Vascular Study. Atherosclerosis. 2011;219:211–7.
48. Santovito D, Mandolini C, Marcantonio P, De Nardis V, Bucci M, Paganelli C, et al. Overexpression of microRNA-145 in atherosclerotic plaques from hypertensive patients. Expert Opin Ther Targets. 2013;17:217–23.

49. Gan CS, Wang CW, Tan KS. Circulatory microRNA-145 expression is increased in cerebral ischemia. Genet Mol Res. 2012;11:147–52.
50. Tsai PC, Liao YC, Wang YS, Lin HF, Lin RT, Juo SH. Serum microRNA-21 and microRNA-221 as potential biomarkers for cerebrovascular disease. J Vasc Res. 2013;50:346–54.
51. Peng H, Zhong M, Zhao W, Wang C, Zhang J, Liu X, et al. Urinary miR-29 correlates with albuminuria and carotid intima-media thickness in type 2 diabetes patients. PLoS One. 2013;8, e82607.
52. Menghini R, Casagrande V, Cardellini M, Martelli E, Terrinoni A, Amati F, et al. MicroRNA 217 modulates endothelial cell senescence via silent information regulator 1. Circulation. 2009;120:1524–32.
53. Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet. 2006;367:1747–57.
54. Feigin VL, Forouzanfar MH, Krishnamurthi R, Mensah GA, Connor M, Bennett DA, et al. Global and regional burden of stroke during 1990-2010: findings from the global burden of disease study 2010. Lancet. 2014;383:245–54.
55. Santulli G. Epidemiology of cardiovascular disease in the 21st century: updated numbers and updated facts. J Cardiovasc Dis. 2013;1:1–2.
56. Menken M, Munsat TL, Toole JF. The global burden of disease study: implications for neurology. Arch Neurol. 2000;57:418–20.
57. Anderson CS, Heeley E, Huang Y, Wang J, Stapf C, Delcourt C, et al. Rapid blood-pressure lowering in patients with acute intracerebral hemorrhage. N Engl J Med. 2013;368:2355–65.
58. Mayer SA, Brun NC, Begtrup K, Broderick J, Davis S, Diringer MN, et al. Efficacy and safety of recombinant activated factor VII for acute intracerebral hemorrhage. N Engl J Med. 2008;358:2127–37.
59. Yuan ZH, Jiang JK, Huang WD, Pan J, Zhu JY, Wang JZ. A meta-analysis of the efficacy and safety of recombinant activated factor VII for patients with acute intracerebral hemorrhage without hemophilia. J Clin Neurosci. 2010;17:685–93.
60. Mendelow AD, Gregson BA, Rowan EN, Murray GD, Gholkar A, Mitchell PM. Early surgery versus initial conservative treatment in patients with spontaneous supratentorial lobar intracerebral haematomas (STICH II): a randomised trial. Lancet. 2013;382:397–408.
61. Soreq H, Wolf Y. NeurimmiRs: microRNAs in the neuroimmune interface. Trends Mol Med. 2011;17:548–55.
62. van Empel VP, De Windt LJ, da Costa Martins PA. Circulating miRNAs: reflecting or affecting cardiovascular disease? Curr Hypertens Rep. 2012;14:498–509.
63. Saikumar J, Ramachandran K, Vaidya VS. Noninvasive micromarkers. Clin Chem. 2014;60:1158–73.
64. Heggermont WA, Heymans S. MicroRNAs are involved in end-organ damage during hypertension. Hypertension. 2012;60:1088–93.
65. Matsumoto S, Sakata Y, Suna S, Nakatani D, Usami M, Hara M, et al. Circulating p53-responsive microRNAs are predictive indicators of heart failure after acute myocardial infarction. Circ Res. 2013;113:322–6.
66. Kim JM, Jung KH, Chu K, Lee ST, Ban J, Moon J, et al. Atherosclerosis-related circulating microRNAs as a predictor of stroke recurrence. Transl Stroke Res. 2015;6(3):191–7.
67. Dharap A, Bowen K, Place R, Li LC, Vemuganti R. Transient focal ischemia induces extensive temporal changes in rat cerebral microRNAome. J Cereb Blood Flow Metab. 2009;29:675–87.
68. Gubern C, Camos S, Ballesteros I, Rodriguez R, Romera VG, Canadas R, et al. miRNA expression is modulated over time after focal ischaemia: up-regulation of miR-347 promotes neuronal apoptosis. FEBS J. 2013;280:6233–46.

69. Jeyaseelan K, Lim KY, Armugam A. MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. Stroke. 2008;39:959–66.
70. Liu FJ, Lim KY, Kaur P, Sepramaniam S, Armugam A, Wong PT, et al. MicroRNAs involved in regulating spontaneous recovery in embolic stroke model. PloS One. 2013;8:e66393.
71. Selvamani A, Williams MH, Miranda RC, Sohrabji F. Circulating miRNA profiles provide a biomarker for severity of stroke outcomes associated with age and sex in a rat model. Clin Sci (Lond). 2014;127:77–89.
72. Weng H, Shen C, Hirokawa G, Ji X, Takahashi R, Shimada K, et al. Plasma miR-124 as a biomarker for cerebral infarction. Biomed Res. 2011;32:135–41.
73. Jickling GC, Ander BP, Zhan X, Noblett D, Stamova B, Liu D. MicroRNA expression in peripheral blood cells following acute ischemic stroke and their predicted gene targets. PloS One. 2014;9, e99283.
74. Sepramaniam S, Tan JR, Tan KS, DeSilva DA, Tavintharan S, Woon FP, et al. Circulating microRNAs as biomarkers of acute stroke. Int J Mol Sci. 2014;15:1418–32.
75. Sorensen SS, Nygaard AB, Nielsen MY, Jensen K, Christensen T. miRNA expression profiles in cerebrospinal fluid and blood of patients with acute ischemic stroke. Transl Stroke Res. 2014;5:711–8.
76. Tan JR, Tan KS, Koo YX, Yong FL, Wang CW, Armugam A, et al. Blood microRNAs in low or no risk ischemic stroke patients. Int J Mol Sci. 2013;14:2072–84.
77. Tan KS, Armugam A, Sepramaniam S, Lim KY, Setyowati KD, Wang CW, et al. Expression profile of microRNAs in young stroke patients. PLoS One. 2009;4, e7689.
78. Wang W, Sun G, Zhang L, Shi L, Zeng Y. Circulating microRNAs as novel potential biomarkers for early diagnosis of acute stroke in humans. J Stroke Cerebrovasc Dis. 2014;23:2607–13.
79. Li P, Teng F, Gao F, Zhang M, Wu J, Zhang C. Identification of circulating microRNAs as potential biomarkers for detecting acute ischemic stroke. Cell Mol Neurobiol. 2015;35(3):433–47.
80. Rehfeld F, Rohde AM, Nguyen DT, Wulczyn FG. Lin28 and let-7: ancient milestones on the road from pluripotency to neurogenesis. Cell Tissue Res. 2015;359:145–60.
81. Selvamani A, Sathyan P, Miranda RC, Sohrabji F. An antagomir to microRNA Let7f promotes neuroprotection in an ischemic stroke model. PLoS One. 2012;7, e32662.
82. Buller B, Liu X, Wang X, Zhang RL, Zhang L, Hozeska-Solgot A, et al. MicroRNA-21 protects neurons from ischemic death. FEBS J. 2010;277:4299–307.
83. Zhou J, Zhang J. Identification of miRNA-21 and miRNA-24 in plasma as potential early stage markers of acute cerebral infarction. Mol Med Rep. 2014;10:971–6.
84. Kole AJ, Swahari V, Hammond SM, Deshmukh M. miR-29b is activated during neuronal maturation and targets BH3-only genes to restrict apoptosis. Genes Dev. 2011;25:125–30.
85. Pandi G, Nakka VP, Dharap A, Roopra A, Vemuganti R. MicroRNA miR-29c downregulation leading to de-repression of its target DNA methyltransferase 3a promotes ischemic brain damage. PLoS One. 2013;8, e58039.
86. Shi G, Liu Y, Liu T, Yan W, Liu X, Wang Y, et al. Upregulated miR-29b promotes neuronal cell death by inhibiting Bcl2L2 after ischemic brain injury. Exp Brain Res. 2012;216:225–30.
87. Huang LG, Li JP, Pang XM, Chen CY, Xiang HY, Feng LB, et al. MicroRNA-29c correlates with neuroprotection induced by FNS by targeting both Birc2 and Bak1 in rat brain after stroke. CNS Neurosci Ther. 2015;21(6):496–503.
88. Dhiraj DK, Chrysanthou E, Mallucci GR, Bushell M. miRNAs-19b, -29b-2* and -339-5p show an early and sustained up-regulation in ischemic models of stroke. PloS One. 2013;8:e83717.
89. Zhu F, Liu JL, Li JP, Xiao F, Zhang ZX, Zhang L. MicroRNA-124 (miR-124) regulates Ku70 expression and is correlated with neuronal death induced by ischemia/reperfusion. J Mol Neurosci. 2014;52:148–55.

90. Liu XS, Chopp M, Zhang RL, Tao T, Wang XL, Kassis H, et al. MicroRNA profiling in subventricular zone after stroke: MiR-124a regulates proliferation of neural progenitor cells through Notch signaling pathway. PLoS One. 2011;6, e23461.
91. Doeppner TR, Doehring M, Bretschneider E, Zechariah A, Kaltwasser B, Muller B, et al. MicroRNA-124 protects against focal cerebral ischemia via mechanisms involving Usp14-dependent REST degradation. Acta Neuropathol. 2013;126:251–65.
92. Sun Y, Gui H, Li Q, Luo ZM, Zheng MJ, Duan JL, et al. MicroRNA-124 protects neurons against apoptosis in cerebral ischemic stroke. CNS Neurosci Ther. 2013;19:813–9.
93. Liu X, Li F, Zhao S, Luo Y, Kang J, Zhao H, et al. MicroRNA-124-mediated regulation of inhibitory member of apoptosis-stimulating protein of p53 family in experimental stroke. Stroke. 2013;44:1973–80.
94. Ouyang YB, Lu Y, Yue S, Xu LJ, Xiong XX, White RE, et al. miR-181 regulates GRP78 and influences outcome from cerebral ischemia in vitro and in vivo. Neurobiol Dis. 2012;45:555–63.
95. Moon JM, Xu L, Giffard RG. Inhibition of microRNA-181 reduces forebrain ischemia-induced neuronal loss. J Cereb Blood Flow Metab. 2013;33:1976–82.
96. Ouyang YB, Lu Y, Yue S, Giffard RG. miR-181 targets multiple Bcl-2 family members and influences apoptosis and mitochondrial function in astrocytes. Mitochondrion. 2012;12:213–9.
97. Xu LJ, Ouyang YB, Xiong X, Stary CM, Giffard RG. Post-stroke treatment with miR-181 antagomir reduces injury and improves long-term behavioral recovery in mice after focal cerebral ischemia. Exp Neurol. 2015;264:1–7.
98. Zeng L, He X, Wang Y, Tang Y, Zheng C, Cai H, et al. MicroRNA-210 overexpression induces angiogenesis and neurogenesis in the normal adult mouse brain. Gene Ther. 2014;21:37–43.
99. Lou YL, Guo F, Liu F, Gao FL, Zhang PQ, Niu X, et al. miR-210 activates notch signaling pathway in angiogenesis induced by cerebral ischemia. Mol Cell Biochem. 2012;370:45–51.
100. Harraz MM, Eacker SM, Wang X, Dawson TM, Dawson VL. MicroRNA-223 is neuroprotective by targeting glutamate receptors. Proc Natl Acad Sci U S A. 2012;109:18962–7.
101. Liu XS, Chopp M, Wang XL, Zhang L, Hozeska-Solgot A, Tang T, et al. MicroRNA-17-92 cluster mediates the proliferation and survival of neural progenitor cells after stroke. J Biol Chem. 2013;288:12478–88.
102. Vinciguerra A, Formisano L, Cerullo P, Guida N, Cuomo O, Esposito A, et al. MicroRNA-103-1 selectively downregulates brain NCX1 and its inhibition by anti-miRNA ameliorates stroke damage and neurological deficits. Mol Ther. 2014;22:1829–38.
103. Yang ZB, Zhang Z, Li TB, Lou Z, Li SY, Yang H, et al. Up-regulation of brain-enriched miR-107 promotes excitatory neurotoxicity through down-regulation of glutamate transporter-1 expression following ischaemic stroke. Clin Sci (Lond). 2014;127:679–89.
104. Chi W, Meng F, Li Y, Li P, Wang G, Cheng H, et al. Impact of microRNA-134 on neural cell survival against ischemic injury in primary cultured neuronal cells and mouse brain with ischemic stroke by targeting HSPA12B. Brain Res. 2014;1592:22–33.
105. Chi W, Meng F, Li Y, Wang Q, Wang G, Han S, et al. Downregulation of miRNA-134 protects neural cells against ischemic injury in N2A cells and mouse brain with ischemic stroke by targeting HSPA12B. Neuroscience. 2014;277:111–22.
106. Zhao L, Li H, Guo R, Ma T, Hou R, Ma X, et al. miR-137, a new target for post-stroke depression? Neural Regen Res. 2013;8:2441–8.
107. Stary CM, Xu L, Sun X, Ouyang YB, White RE, Leong J, et al. MicroRNA-200c contributes to injury from transient focal cerebral ischemia by targeting reelin. Stroke. 2015;46:551–6.
108. Li LJ, Huang Q, Zhang N, Wang GB, Liu YH. miR-376b-5p regulates angiogenesis in cerebral ischemia. Mol Med Rep. 2014;10:527–35.
109. Zhao H, Wang J, Gao L, Wang R, Liu X, Gao Z, et al. MiRNA-424 protects against permanent focal cerebral ischemia injury in mice involving suppressing microglia activation. Stroke. 2013;44:1706–13.

110. Liu P, Zhao H, Wang R, Wang P, Tao Z, Gao L, et al. MicroRNA-424 protects against focal cerebral ischemia and reperfusion injury in mice by suppressing oxidative stress. Stroke. 2015;46:513–9.
111. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature. 2000;408:86–9.
112. Hulsmans M, Holvoet P. MicroRNA-containing microvesicles regulating inflammation in association with atherosclerotic disease. Cardiovasc Res. 2013;100:7–18.
113. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9:654–9.
114. Bienertova-Vasku J, Novak J, Vasku A. MicroRNAs in pulmonary arterial hypertension: pathogenesis, diagnosis and treatment. J Am Soc Hypertens. 2015;9(3):221–34.
115. Dong S, Cheng Y, Yang J, Li J, Liu X, Wang X, et al. MicroRNA expression signature and the role of microRNA-21 in the early phase of acute myocardial infarction. J Biol Chem. 2009;284:29514–25.
116. Khanna S, Rink C, Ghoorkhanian R, Gnyawali S, Heigel M, Wijesinghe DS, et al. Loss of miR-29b following acute ischemic stroke contributes to neural cell death and infarct size. J Cereb Blood Flow Metab. 2013;33:1197–206.
117. Kriegel AJ, Liu Y, Fang Y, Ding X, Liang M. The miR-29 family: genomics, cell biology, and relevance to renal and cardiovascular injury. Physiol Genomics. 2012;44:237–44.
118. Meza-Sosa KF, Pedraza-Alva G, Perez-Martinez L. MicroRNAs: key triggers of neuronal cell fate. Front Cell Neurosci. 2014;8:175.
119. Laterza OF, Lim L, Garrett-Engele PW, Vlasakova K, Muniappa N, Tanaka WK, et al. Plasma microRNAs as sensitive and specific biomarkers of tissue injury. Clin Chem. 2009;55:1977–83.
120. Luo X, Hou L, Shi H, Zhong X, Zhang Y, Zheng D, et al. CSF levels of the neuronal injury biomarker visinin-like protein-1 in Alzheimer's disease and dementia with Lewy bodies. J Neurochem. 2013;127:681–90.
121. Fang M, Wang J, Zhang X, Geng Y, Hu Z, Rudd JA, et al. The miR-124 regulates the expression of BACE1/beta-secretase correlated with cell death in Alzheimer's disease. Toxicol Lett. 2012;209:94–105.
122. Su W, Hopkins S, Nesser NK, Sopher B, Silvestroni A, Ammanuel S, et al. The p53 transcription factor modulates microglia behavior through microRNA-dependent regulation of c-Maf. J Immunol. 2014;192:358–66.
123. Miska EA, Alvarez-Saavedra E, Townsend M, Yoshii A, Sestan N, Rakic P, et al. Microarray analysis of microRNA expression in the developing mammalian brain. Genome Biol. 2004;5:R68.
124. Su R, Lin HS, Zhang XH, Yin XL, Ning HM, Liu B, et al. MiR-181 family: regulators of myeloid differentiation and acute myeloid leukemia as well as potential therapeutic targets. Oncogene. 2015;34(25):3226–39.
125. Ouyang YB, Giffard RG. MicroRNAs affect BCL-2 family proteins in the setting of cerebral ischemia. Neurochem Int. 2014;77:2–8.
126. Ouyang YB, Giffard RG. MicroRNAs regulate the chaperone network in cerebral ischemia. Transl Stroke Res. 2013;4:693–703.
127. Ouyang YB, Stary CM, White RE, Giffard RG. The use of microRNAs to modulate redox and immune response to stroke. Antioxid Redox Signal. 2015;22:187–202.
128. Ouyang YB, Xu L, Liu S, Giffard RG. Role of astrocytes in delayed neuronal death: GLT-1 and its novel regulation by microRNAs. Adv Neurobiol. 2014;11:171–88.
129. Chan YC, Banerjee J, Choi SY, Sen CK. miR-210: the master hypoxamir. Microcirculation. 2012;19:215–23.
130. Fasanaro P, D'Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, et al. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. J Biol Chem. 2008;283:15878–83.

131. Sepramaniam S, Ying LK, Armugam A, Wintour EM, Jeyaseelan K. MicroRNA-130a represses transcriptional activity of aquaporin 4 M1 promoter. J Biol Chem. 2012;287:12006–15.
132. Sepramaniam S, Armugam A, Lim KY, Karolina DS, Swaminathan P, Tan JR, et al. MicroRNA 320a functions as a novel endogenous modulator of aquaporins 1 and 4 as well as a potential therapeutic target in cerebral ischemia. J Biol Chem. 2010;285:29223–30.
133. Pan Y, Liang H, Liu H, Li D, Chen X, Li L, et al. Platelet-secreted microRNA-223 promotes endothelial cell apoptosis induced by advanced glycation end products via targeting the insulin-like growth factor 1 receptor. J Immunol. 2014;192:437–46.
134. Tabet F, Vickers KC, Cuesta Torres LF, Wiese CB, Shoucri BM, Lambert G, et al. HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. Nat Commun. 2014;5:3292.
135. Vickers KC, Landstreet SR, Levin MG, Shoucri BM, Toth CL, Taylor RC, et al. MicroRNA-223 coordinates cholesterol homeostasis. Proc Natl Acad Sci U S A. 2014;111:14518–23.
136. Duan X, Zhan Q, Song B, Zeng S, Zhou J, Long Y, et al. Detection of platelet microRNA expression in patients with diabetes mellitus with or without ischemic stroke. J Diabetes Complications. 2014;28:705–10.
137. Mogilyansky E, Rigoutsos I. The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. Cell Death Differ. 2013;20:1603–14.
138. Leung LY, Chan CP, Leung YK, Jiang HL, Abrigo JM, de Wang F, et al. Comparison of miR-124-3p and miR-16 for early diagnosis of hemorrhagic and ischemic stroke. Clin Chim Acta. 2014;433:139–44.
139. Liu Y, Zhang J, Han R, Liu H, Sun D, Liu X. Downregulation of serum brain specific microRNA is associated with inflammation and infarct volume in acute ischemic stroke. J Clin Neurosci. 2015;22:291–5.
140. Long G, Wang F, Li H, Yin Z, Sandip C, Lou Y, et al. Circulating miR-30a, miR-126 and let-7b as biomarker for ischemic stroke in humans. BMC Neurol. 2013;13:178.
141. Zeng L, Liu J, Wang Y, Wang L, Weng S, Tang Y, et al. MicroRNA-210 as a novel blood biomarker in acute cerebral ischemia. Front Biosci (Elite Ed). 2011;3:1265–72.
142. Zeng L, Liu J, Wang Y, Wang L, Weng S, Chen S, et al. Cocktail blood biomarkers: prediction of clinical outcomes in patients with acute ischemic stroke. Eur Neurol. 2013;69:68–75.
143. Wang Y, Zhang Y, Huang J, Chen X, Gu X, Zeng L, et al. Increase of circulating miR-223 and insulin-like growth factor-1 is associated with the pathogenesis of acute ischemic stroke in patients. BMC Neurol. 2014;14:77.
144. Brown Jr RD, Broderick JP. Unruptured intracranial aneurysms: epidemiology, natural history, management options, and familial screening. Lancet Neurol. 2014;13(4):393–404.
145. Brown Jr RD, Wiebers DO, Forbes GS. Unruptured intracranial aneurysms and arteriovenous malformations: frequency of intracranial hemorrhage and relationship of lesions. J Neurosurg. 1990;73:859–63.
146. Shiue I, Arima H, Hankey GJ, Anderson CS. Modifiable lifestyle behaviours account for most cases of subarachnoid haemorrhage: a population-based case-control study in Australasia. J Neurol Sci. 2012;313:92–4.
147. Vernooij MW, Ikram MA, Tanghe HL, Vincent AJ, Hofman A, Krestin GP, et al. Incidental findings on brain MRI in the general population. N Engl J Med. 2007;357:1821–8.
148. Molyneux AJ, Birks J, Clarke A, Sneade M, Kerr RS. The durability of endovascular coiling versus neurosurgical clipping of ruptured cerebral aneurysms: 18 year follow-up of the UK cohort of the International subarachnoid Aneurysm Trial (ISAT). Lancet. 2015; 385(9969):691–7.
149. Lee HJ, Yi JS, Lee IW, Park KC, Yang JH. Dysregulated expression profiles of microRNAs of experimentally induced cerebral aneurysms in rats. J Korean Neurosurg Soc. 2013;53: 72–6.
150. Jiang Y, Zhang M, He H, Chen J, Zeng H, Li J, et al. MicroRNA/mRNA profiling and regulatory network of intracranial aneurysm. BMC Med Genomics. 2013;6:36.

151. Liu D, Han L, Wu X, Yang X, Zhang Q, Jiang F. Genome-wide microRNA changes in human intracranial aneurysms. BMC Neurol. 2014;14:188.
152. Jin H, Li C, Ge H, Jiang Y, Li Y. Circulating microRNA: a novel potential biomarker for early diagnosis of intracranial aneurysm rupture a case control study. J Transl Med. 2013;11:296.
153. Li P, Zhang Q, Wu X, Yang X, Zhang Y, Li Y, et al. Circulating microRNAs serve as novel biological markers for intracranial aneurysms. J Am Heart Assoc. 2014;3, e000972.
154. Maegdefessel L, Azuma J, Toh R, Deng A, Merk DR, Raiesdana A, et al. MicroRNA-21 blocks abdominal aortic aneurysm development and nicotine-augmented expansion. Sci Transl Med. 2012;4:122ra22.
155. Leeper NJ, Raiesdana A, Kojima Y, Chun HJ, Azuma J, Maegdefessel L, et al. MicroRNA-26a is a novel regulator of vascular smooth muscle cell function. J Cell Physiol. 2011;226:1035–43.
156. Boon RA, Seeger T, Heydt S, Fischer A, Hergenreider E, Horrevoets AJ, et al. MicroRNA-29 in aortic dilation: implications for aneurysm formation. Circ Res. 2011;109:1115–9.
157. Maegdefessel L, Azuma J, Toh R, Merk DR, Deng A, Chin JT, et al. Inhibition of microRNA-29b reduces murine abdominal aortic aneurysm development. J Clin Invest. 2012;122:497–506.
158. Merk DR, Chin JT, Dake BA, Maegdefessel L, Miller MO, Kimura N, et al. miR-29b participates in early aneurysm development in Marfan syndrome. Circ Res. 2012;110:312–24.
159. Tan J, Tong BD, Wu YJ, Xiong W. MicroRNA-29 mediates TGFbeta1-induced extracellular matrix synthesis by targeting wnt/beta-catenin pathway in human orbital fibroblasts. Int J Clin Exp Pathol. 2014;7:7571–7.
160. Abecassis IJ, Xu DS, Batjer HH, Bendok BR. Natural history of brain arteriovenous malformations: a systematic review. Neurosurg Focus. 2014;37:E7.
161. Hofmeister C, Stapf C, Hartmann A, Sciacca RR, Mansmann U, terBrugge K, et al. Demographic, morphological, and clinical characteristics of 1289 patients with brain arteriovenous malformation. Stroke. 2000;31:1307–10.
162. Mohr JP, Parides MK, Stapf C, Moquete E, Moy CS, Overbey JR, et al. Medical management with or without interventional therapy for unruptured brain arteriovenous malformations (ARUBA): a multicentre, non-blinded, randomised trial. Lancet. 2014;383:614–21.
163. Stapf C, Mohr JP, Pile-Spellman J, Solomon RA, Sacco RL, Connolly Jr ES. Epidemiology and natural history of arteriovenous malformations. Neurosurg Focus. 2001;11(5):e1.
164. Gross BA, Du R. Natural history of cerebral arteriovenous malformations: a meta-analysis. J Neurosurg. 2013;118:437–43.
165. Stefani MA, Porter PJ, terBrugge KG, Montanera W, Willinsky RA, Wallace MC. Large and deep brain arteriovenous malformations are associated with risk of future hemorrhage. Stroke. 2002;33:1220–4.
166. Stefani MA, Porter PJ, terBrugge KG, Montanera W, Willinsky RA, Wallace MC. Angioarchitectural factors present in brain arteriovenous malformations associated with hemorrhagic presentation. Stroke. 2002;33:920–4.
167. Ferreira R, Santos T, Amar A, Gong A, Chen TC, Tahara SM, et al. Argonaute-2 promotes miR-18a entry in human brain endothelial cells. J Am Heart Assoc. 2014;3, e000968.
168. Ferreira R, Santos T, Amar A, Tahara SM, Chen TC, Giannotta SL, et al. MicroRNA-18a improves human cerebral arteriovenous malformation endothelial cell function. Stroke. 2014;45:293–7.

Chapter 10
microRNAs and Cardiovascular Remodeling

Koh Ono

Abstract Heart failure (HF) is associated with significant morbidity and mortality attributable largely to structural changes in the heart and with associated cardiac dysfunction. Remodeling is defined as alteration of the mass, dimensions, or shape of the heart (termed cardiac or ventricular remodeling) and vessels (vascular remodeling) in response to hemodynamic load and/or cardiovascular injury in association with neurohormonal activation. Remodeling may be described as physiologic or pathologic; alternatively, remodeling may be classified as adaptive or maladaptive. The importance of remodeling as a pathogenic mechanism has been controversial because factors leading to remodeling as well as the remodeling itself may be major determinants of patients' prognosis. The basic mechanisms of cardiovascular remodeling, and especially the roles of microRNAs in HF progression and vascular diseases, will be reviewed here.

Keywords Hypertrophy • Ischemia • Fibrosis • Heart failure • Atherosclerosis

Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in developed countries. Cardiovascular remodeling is thought to be an important aspect of disease progression in heart failure (HF), regardless of cause. It is manifested clinically by changes in cardiac size, shape, and function in response to aging, cardiac injury, or increased load. The importance of remodeling as a pathogenic mechanism is not completely understood because the factors leading to remodeling have not been fully investigated. Generally, pathological processes of the heart are associated with an altered expression profile of genes that are important for cardiac function (Fig. 10.1) [1].

K. Ono (✉)
Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
e-mail: kohono@kuhp.kyoto-u.ac.jp

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_10

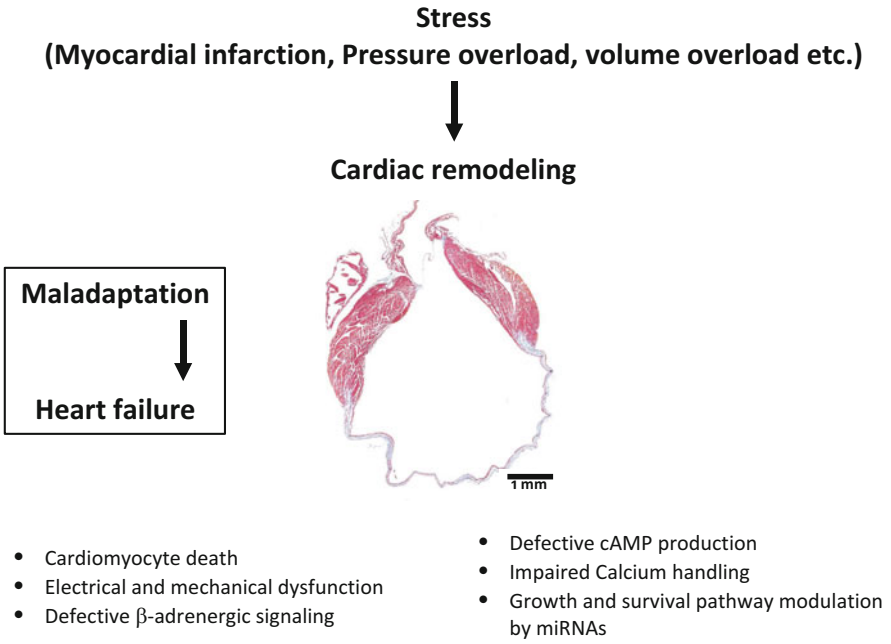

Fig. 10.1 Pathological processes of the heart under stress

The regulation of cardiac gene expression is complex, with individual genes controlled by multiple transcription factors associated with their regulatory enhancer/promoter sequences to activate gene expression [2]. Moreover, epigenetic regulation of gene expression and alternative splicing mechanisms also further complicate the patterns of gene expression. microRNAs (miRNAs; miRs) have reshaped our view of how gene expression is regulated by adding another layer of regulation at the posttranscriptional level. Cardiovascular remodeling encompassed many pathologies including cardiac hypertrophy, myocardial ischemia/myocardial infarction (MI), cardiac fibrosis, arrhythmia, and vascular diseases that will be discussed in more detail in the following sections (Fig. 10.2).

The implications of miRNA-derived regulation in cardiovascular pathology have only been recognized very recently, and research on miRNAs in relation to such diseases has now become a rapidly evolving field. In this chapter, we will summarize the current understanding of miRNA function in the pathogenesis of cardiovascular remodeling.

Cardiac Hypertrophy

Left ventricular hypertrophy is a common finding in patients with hypertension and it can be diagnosed either using an electrocardiogram or by echocardiography. Because cardiac hypertrophy, an increase in heart size, is associated with nearly all

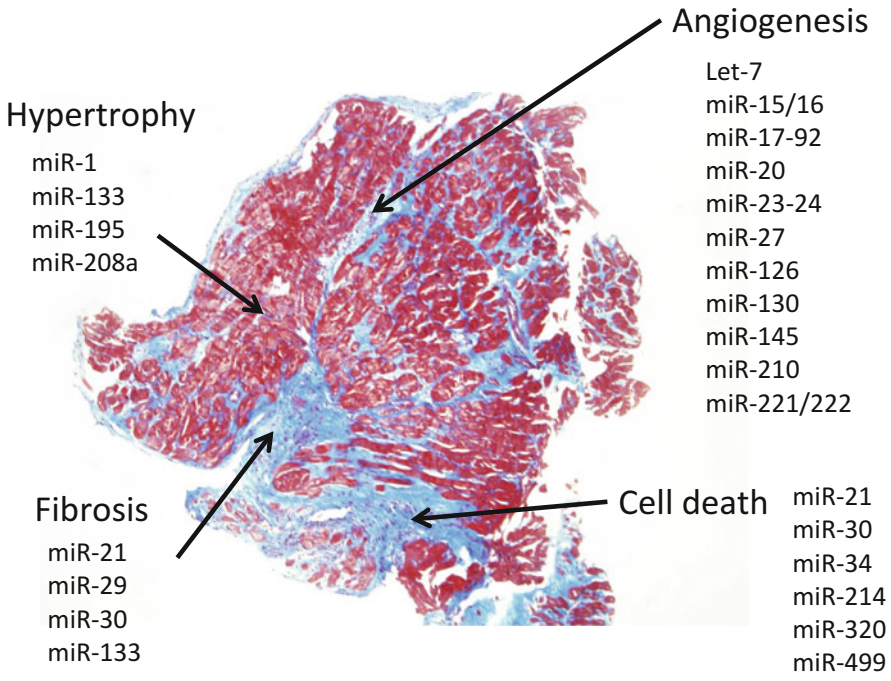

Fig. 10.2 Dysregulated microRNAs in cardiovascular disease

forms of HF, it is of great clinical importance that we understand the mechanisms responsible for cardiac hypertrophy. Therefore, the regulation of hypertrophy-associated genes has attracted great interest from many researchers.

Pathological hypertrophy is mainly caused by hypertension, loss of myocytes following ischemic damage, and genetic alterations that cause cardiomyopathy. Moreover, metabolic abnormality or neurohormonal activation can also lead to hypertrophy [3]. Pathological hypertrophy is the phenotypic endpoint that has been most studied in relation to miRNAs in the heart to date.

Clinical studies in human hearts have indicated that the fetal gene expression program is reactivated in pathologic hypertrophy and failing hearts, which results in a switching of structural proteins from adult to fetal isoforms [4]. It is well known that there is a decrease in the fast-shortening-velocity isoform (α-myosin heavy chain) coupled with an increase in the slow-shortening-velocity isoform (β-myosin heavy chain [β-MHC]) during the transition from cardiac hypertrophy to HF [5]. This contributes to the decrease in contractile function in failing human hearts. Interestingly, transcriptome analysis of failing and fetal hearts revealed a similar pattern of miRNA expression. More than 80 % of the deregulated miRNAs in failing hearts displayed a similar expression pattern as in fetal hearts, suggesting that reactivation of a fetal miRNA program may contribute to the gene expression pattern of failing hearts [6]. The most consistent changes were upregulation of miR-21,

miR-29b, miR-129, miR-210, miR-211, miR-212, and miR-423, with downregulation of miR-30, miR-182, and miR-526. Interestingly, gene expression analysis revealed that most of the upregulated genes were characterized by the presence of a significant number of the predicted binding sites for downregulated miRNAs and vice versa.

In animal models of cardiac hypertrophy, whole arrays of miRNAs have indicated that separate miRNAs are upregulated, downregulated, or remain unchanged with respect to their levels in a normal heart [6–12]. In these studies, some miRNAs have been more frequently reported than others, indicating the possibility that these miRNAs might have common roles in hypertrophy pathogenesis.

Tissue-specific expression of miRNAs was first reported in 2002 [13]. It is known that there is a family of so-called myomiRs that are encoded within the introns of the separate myosin heavy chain genes. miR-208a, miR-208b, and miR-499 are located within the Myh6, Myh7, and Myh7b genes, respectively. It was reported that miR-208$^{-/-}$ mice show reduced cardiac hypertrophy in response to pressure overload [14]. Targets of miR-208a include thyroid hormone receptor-associated protein 1 [14, 15], suggesting that miR-208a initiates cardiomyocyte hypertrophy by regulating triiodothyronine-dependent repression of β-MHC expression. miR-27a also regulates β-MHC gene expression by targeting TRβ1 in cardiomyocytes [16]. Overexpression of miR-208a was sufficient to upregulate Myh7 and to elicit cardiac hypertrophy, resulting in systolic dysfunction [15]. Although miR-208a is required for cardiac hypertrophy, the role of miR-208b in these pathologic conditions remains to be elucidated. miR-499 is encoded in an intron of the myh7 gene and is considered likely to play a role in myosin gene regulation [17, 18].

miR-1 is also a cardiac and skeletal muscle-specific miRNA, and it is probably one of the most abundant miRNAs in the heart. It was reported to target a cytoskeletal regulatory protein, twinfilin 1 (Twf1), which binds to actin monomers and prevents their assembly into filaments [19]. Downregulation of miR-1 induced by hypertrophic stimuli, such as transverse aortic constriction or α-adrenergic stimulation with phenylephrine, results in increased Twf1 expression, and overexpression of Twf1 is sufficient to induce cardiac hypertrophy. Another target of miR-1 is insulin-like growth factor (IGF-1), IGF-1 receptor [20], calmodulin 1 and 2, Mef2a [21], and sodium calcium exchanger [22]. Repression of miR-1 and upregulation of IGF-1 was also demonstrated in models of cardiac hypertrophy [20]. miR-1 is downregulated in patients with aortic stenosis [11] and acromegaly associated cardiac hypertrophy [20].

miR-1 is encoded by two bicistronic clusters—miR-133a-1/miR-1-2 and miR-133a-2/miR-1-1. As well as miR-1, miR-133 also has the potential to attenuate agonist-induced hypertrophy [23, 24], whereas repression of miR-133 sensitized the myocardium to excessive cardiac growth. Therefore, these clusters generate antagonizing effects on the stimulation of cardiac hypertrophy.

In contrast, miR-195 was sufficient to drive pathologic cardiac growth when overexpressed in neonatal cardiac myocytes and in transgenic mice [7]. These results suggested that miR-195 is a pro-hypertrophic factor that actively participates in the hypertrophic process; however, no direct targets of miR-195 have been reported in the context of cardiac hypertrophy.

Myocardial Ischemia and Cell Death

A rapidly increasing number of studies have shown that cardiac and circulating miRNAs are markedly altered in myocardial ischemia or MI. These novel findings shed new light on the mechanisms that lead to MI complications, post-MI ventricular remodeling, and cardiac repair. Furthermore, recent studies showed that circulating miRNAs may represent novel and sensitive biomarkers of MI and, possibly, also function as an intercellular signaling mechanism (see Chap. 7 of the volume "microRNA: Basic Science" for a detailed discussion of miRNA and cardiac regeneration).

Cardiomyocyte death/apoptosis is a key cellular event in ischemic hearts. There are miRNAs that have been shown to exert proapoptotic effects by targeting key cardioprotective proteins. It was found that miR-320 expression was consistently dysregulated in ischemic hearts [25]. Ren et al. identified heat-shock protein 20 (HSP20), a known cardioprotective protein, as a target of miR-320. Knockdown of endogenous miR-320 provided protection against cardiomyocyte apoptosis through the upregulation of HSP20. miR-34 family members promote growth arrest and apoptosis [26]. Therapeutic inhibition of miR-34 attenuated ischemia-induced remodeling and improved cardiac recovery [27]. One of the targets of miR-34 was shown to be a protein phosphatase 1 nuclear targeting subunit (Pnuts) [28].

On the other hand, there are a number of miRNAs that exert an antiapoptotic function by targeting important proapoptotic proteins. The miRNA expression signature in rat hearts at 6 h after MI revealed that miR-21 expression was significantly downregulated in infracted areas but was upregulated in border areas [29]. Adenoviral transfer of miR-21 in vivo decreased cell apoptosis in the border and infracted areas through its target gene, programmed cell death 4 (PDCD4), and the activator protein 1 (AP1) pathway. miR-24 also inhibited cardiomyocyte apoptosis via repression of the proapoptotic protein Bim [30]. Ex vivo miR-24 enrichment, together with miR-21 and miR-221, improved the therapeutic potential of cardiac progenitor cells upon transplantation in ischemic rodents [31]. Similarly, miR-499 and miR-30 family members diminished apoptosis in injured hearts by attenuating activation of dynamin-related protein-1 and thus inhibiting mitochondrial fission [32, 33].

Early reperfusion of the ischemic heart remains the most effective intervention for improving clinical outcomes after a MI. However, abnormal increases in intracellular Ca^{2+} during myocardial reperfusion can cause cardiomyocyte death, known as ischemia-reperfusion (I/R) injury. Cardiac I/R injury is also accompanied by dynamic changes in the expression of miRNAs; for example, miR-214 is upregulated during ischemic injury. Genetic deletion of miR-214 in mice caused a loss of cardiac contractility, increased apoptosis, and excessive fibrosis in response to I/R injury [34]. The cardioprotective roles of miR-214 during I/R injury were attributed to repression of the mRNA encoding sodium/calcium exchanger 1, a key regulator of Ca^{2+} influx; and to repression of several downstream effectors of Ca^{2+} signaling that mediate cell death. These results suggested a pivotal role for miR-214 as a regulator of cardiomyocyte Ca^{2+} homeostasis and survival during cardiac injury. Moreover, CaMKIIδ is a shared target of both miR-214 and miR-145 [35]. miR-145 concomi-

tantly protects cardiomyocytes from reactive oxygen species by targeting Bnip3 [36]. Boosting miR-214 and miR-145 levels to attenuate Ca^{2+} overload and cardiac cell death may provide a valuable therapeutic benefit for the treatment or prevention of heart failure after I/R injury.

Recent studies have shown that some miRNAs are present in circulating blood and that they are included in exosomes and microparticles [37, 38]. Recently, results obtained in studies of cancer suggest that the profiles of blood circulating miRNAs might reflect the changes observed in cancerous tissue [39]. This concept has also proved valid in cardiovascular disease [40], and circulating specific miRNAs have been reported in patients with MI [41, 42]. Moreover, plasma levels of endothelial cell-enriched miRNAs, such as miR-126, miR-17, and miR-92a, inflammation-associated miR-155, and smooth muscle-enriched miR-145 were reported to be significantly reduced in coronary artery disease (CAD) patients compared with healthy controls. These results also indicated that they can be used as biomarker candidates for CAD [43]. Therefore, the source and the mechanism of the change determined the set of miRNAs that can be used for myocardial ischemia/MI.

Cardiac Fibrosis

Cardiac fibrosis is a major aspect of myocardial remodeling and an important contributor to the development of cardiac dysfunction in diverse pathologic conditions, such as MI, in ischemic, dilated, and hypertrophic cardiomyopathies, and HF [44–49]. The extracellular deposition of collagen by fibroblasts contributes to this adverse remodeling. Cardiac fibrosis leads to an increased mechanical stiffness, initially causing diastolic dysfunction, and eventually resulting in systolic dysfunction and overt HF. In addition, fibrosis can also disturb the electrical continuity between cardiomyocytes, leading to conduction slowing and hence an increase in the chance of arrhythmias. It is also possible that the enhanced diffusion distance for cardiac substrates and oxygen to cardiac myocytes, caused by fibrosis, negatively influences the myocardial balance between energy demand and supply [46, 47].

miR-21 is expressed in all cell types of the cardiovascular system, most prominently in cardiac fibroblasts but rather weakly in cardiomyocytes. Furthermore, miR-21 is among the most strongly upregulated miRNAs in response to a variety of forms of cardiac stress [7, 50, 51]. Thum et al. showed that miR-21 is upregulated in cardiac fibroblasts in the failing heart, where it represses the expression of Sprouty homolog 1 (SPRY1), a negative regulator of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK-MAPK) signaling pathway [52]. Upregulation of miR-21 in response to cardiac injury was shown to enhance ERK-MAPK signaling, leading to fibroblast proliferation and fibrosis. Moreover, miR-21-dependent targeting of SPRY1 and PDCD4 was shown to promote the fibroblastoid phenotype in epicardial-to-mesenchymal transition [53]. Phosphatase and tensin homologue (PTEN) has also been demonstrated to be a direct target of miR-21 in cardiac fibroblasts [54]. Previous reports characterized

PTEN as a suppressor of matrix metalloprotease-2 (MMP-2) expression [55, 56]. I/R injury in the heart induced miR-21 in cardiac fibroblasts in the infracted region. Thus, I/R injury-induced miR-21-limited PTEN function and caused activation of the Akt pathway and increased MMP-2 expression in cardiac fibroblasts.

On the other hand, miR-29 family members, miR-133, and miR-30 directly downregulated key profibrotic proteins. The miR-29 family is composed of three members, miR-29a, b, and c. It was shown that the miR-29 family, which is highly expressed in fibroblasts, targets mRNAs encoding a multitude of extracellular matrix (ECM)-related proteins involved in fibrosis, including col1a1, col3a1, elastin, and fibrillin [50]. miR-29 was dramatically repressed in the border zone flanking the infracted area in a mouse model of MI. Downregulation of miR-29 would be predicted to counter the repression of these mRNAs and enhance fibrotic responses. Therefore, it is tempting to speculate that upregulation of miR-29 may be a therapeutic option for MI.

Connective tissue growth factor (CTGF), a key molecule involved in fibrosis, was shown to be regulated by miR-133 and miR-30, which are both consistently downregulated in several models of pathologic hypertrophy and HF [57]. The authors indicated that miR-133 and miR-30 are downregulated during cardiac disease, which inversely correlated with the upregulation of CTGF. In vitro experiments designed to overexpress or inhibit these miRNAs can effectively repress CTGF expression by interacting directly with the 3′-untranslated region (UTR) region of the CTGF mRNA.

Together, these data indicate that miRNAs are important regulators of cardiac fibrosis and are involved in structural heart disease.

Arrhythmias

One of the earliest reports of involvement of miRNA regulation of cardiac repolarization came from Zhou et al. in 2007 with the targeted deletion of miR-1-2 in mice. Surface electrocardiography in mutant mice demonstrated reduced average heart rate, accelerated atrioventricular conduction, and slowed ventricular conduction [58]. They found Irx5 as a target for miR-1-2, which belongs to the Iroquois family of homeodomain-containing transcription factors that regulate cardiac repolarization by repressing transcription of KCND2. KCND2 encodes a K^+ channel subunit (Kv4.2) responsible for the transient outward K^+ current (I_{to}) that is the major determinant of the transmural repolarization gradient in the ventricular wall. The increase in Irx5 protein levels in miR-1-2 mutants corresponded with a decrease in KCND2 expression.

Additional evidence supporting a role for miR-1 in cardiac repolarization and arrhythmogenesis came from a rat model of MI induced by occlusion of the coronary artery. It was established that gap junction protein a1 (GJA1; encoding connexin43 [Cx43]) and potassium inwardly rectifying channel, subfamily J, member 2 (KCNJ2; encoding the K^+ channel subunit Kir2.1) are target genes for miR-1 [59].

Cx43 is critical for inter-cell conductance of excitation [60–62], and Kir2.1 governs cardiac membrane potential [63, 64], both of which are important determinants for cardiac excitability.

To date, the cardiac ion channel genes that have been confirmed experimentally to be targets of miR-1 or miR-133 include GJA1/Cx43/IJ [59], KCNJ2/Kir2.1/IK1 [59], potassium voltage-gated channel, subfamily H (eag-related) member 2 (KCNH2)/human ether-à-go-go-related gene (HERG)/IKr [65], potassium voltage-gated channel, KQT-like subfamily, member 1 (KCNQ1)/KvLQT1/IKs [66], and potassium voltage-gated channel, Isk-related family, member 1 (KCNE1)/mink/IKs [66]. The fact that altered expression of miRNAs can deregulate expression of cardiac ion channels provided novel insight into the molecular understanding of cardiac excitability.

miR-212 has been found to be upregulated in both animal models and human HF [6]. KCNJ2/Kir2.1 3′-UTR contains potential miR-212 binding sites and transfection of HeLa cells with miR-212 reduced inward rectifier K^+ current density, as demonstrated by whole-cell patch-clamp recordings [67].

It was also reported that miR-328 is upregulated in the atria of dogs with induced atrial fibrillation (AF) and targets the L-type calcium channel [68]. Strikingly, inhibition of miR-328 levels with an antagomir reversed the conditions. The fact that genetic knockdown of endogenous miR-328 reduced AF vulnerability also suggests the potential of miR-328 as a target for AF treatment.

Circulating miRNAs, which can be potential biomarkers for AF, were also sought. Plasma miR-150 levels from AF patients were substantially lower than that from healthy people in a cohort of 105 participants [69]. miRNAs may serve as molecular diagnostic markers for AF in the future.

Angiogenesis and Vascular Disease

miRNAs are also important in vascular development, physiology, and disease. Initial evidence for the functional roles of miRNAs in vascular development was provided by the observation that mice carrying a Dicer hypomorphic allele died prenatally with severely disrupted blood vessel formation [70].

Profiling of endothelially expressed miRNAs has been performed using human umbilical vein endothelial cells. These results revealed high expression levels of miR-221/222, miR-21, let-7 family, miR-17-92 cluster, miR-23-24 cluster, and miR-126 in vascular endothelial cells. Among them, miR-126 is the only miRNA considered to be expressed specifically in endothelial cells [71].

Studies focusing on individual miRNAs or miRNA clusters suggested the importance of miRNAs in endothelial cell function and angiogenesis. The miR-17-92 cluster is one of the most important miRNAs for the regulation of angiogenesis. It encodes six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1), which are tightly grouped within an 800 base-pair region, and it is transcriptionally regulated by c-Myc [72]. In particular, miR-18 preferentially suppressed

CTGF, whereas miR-19 targeted the potent angiogenesis-inhibitor thrombospondin-1 to promote tumor angiogenesis [73]. On the other hand, miR-92a controlled the growth of new blood vessels (angiogenesis) [74]. Forced overexpression of miR-92a in endothelial cells blocked angiogenesis, and systemic administration of an antagomir to inhibit miR-92a led to enhanced blood vessel growth and functional recovery of damaged tissue in mouse models of limb ischemia and MI. Therefore, miR-92a may serve as a valuable therapeutic target in the setting of ischemic disease.

miR-126 is an abundant, endothelial cell-enriched miRNA that is encoded in the second intron of an endothelial cell-specific gene, *Egfl7*, and mechano-sensitive zinc finger transcription factor Klf2a was shown to induce miR-126 expression to activate vascular endothelial growth factor signaling [75]. This work described a novel genetic mechanism in which a miRNA facilitated integration of a physiological stimulus with growth factor signaling in endothelial cells to guide angiogenesis. On the other hand, transfection of endothelial cells with an oligonucleotide that decreased miR-126 permitted an increase in tumor necrosis factor-α stimulated vascular adhesion molecule 1 expression and increased leukocyte adherence to endothelial cells [76]. The apparent role of miR-126 in angiogenesis has led to increasing interest in miR-126 overexpression as a therapeutic approach. It has been reported that systemic delivery of miR-126 by miRNA-loaded bubble liposomes improved blood flow and may be useful for the treatment of hind-limb ischemia [77].

There is increasing evidence that specific miRNAs are involved in angiogenesis. So far, pro-angiogenic miRNAs include let7f and miR-27b [78], miR-17-92 cluster [73], miR-126 [79, 80], miR-130a [81], miR-210, and miR-378 [82, 83]. miRNAs that exert anti-angiogenic effects include miR-15/16 [84, 85], miR-20a/b [84], miR-92a [74], and miR-221/222 [86, 87].

In the context of vascular remodeling, Ji et al. identified miRNAs that are aberrantly expressed in the vascular walls after balloon injury [88]. Modulating an aberrantly overexpressed miR-21 via antisense-mediated depletion had a significant negative effect on neointimal lesion formation. They also demonstrated that Pten and Bcl2 were involved in miR-21-mediated cellular effects. The same group also revealed that miR-221 and miR-222 expression levels were elevated in rat carotid arteries after angioplasty [89]. Moreover, they found that p27 (Kip1) and p57 (Kip2) were target genes involved in miR-221- and miR-222-mediated effects on vascular smooth muscle cell (VSMC) growth. Knockdown of miR-221 and miR-222 resulted in decreased VSMC proliferation both in vitro and in vivo.

miR-145 is selectively expressed in VSMCs of the vascular wall, and its expression was significantly downregulated in vascular walls with neointimal lesion formation. The target of miR-145 is KLF5 and its downstream signaling molecule, myocardin. Restoration of miR-145 in balloon-injured arteries via Ad-miR-145 inhibited neointimal growth and might be used for treatment of a variety of proliferative vascular disorders.

Aortic aneurysms are a common clinical condition that can cause death due to aortic dissection or rupture. The association between aortic aneurysm pathogenesis and altered TGF-β signaling, inflammation and apoptosis has been the subject of

numerous investigations. Recently, a TGF-β-responsive miR-29 [90, 91] and miR-21 [92] whose targets include Pten, Spry1, Pdcd4, and Bcl2 have been identified to play roles in cellular phenotypic modulation during aortic development. It was demonstrated that decreasing the levels of miR-29b or increasing the levels of miR-21 in the aortic wall could attenuate aortic aneurysm progression in a porcine pancreatic elastase infusion and angiotensin II infusion model of abdominal aortic aneurysms in mice [90, 92].

Heart Failure

Because all of the previously described pathologies, i.e. cardiac hypertrophy, fibrosis, arrhythmia, and CAD can cause HF, all of the miRNAs discussed so far are also relevant to this disease entity.

Many profiling studies have been conducted and revealed a large number of miRNAs that are differentially expressed in HF, pointing to a new mode of regulation of cardiovascular diseases [9, 11, 12, 21, 57, 93].

A diverse range of circulating miRNAs have been studied for the detection of HF. Tijsen et al. tried to determine whether miRNAs make it possible to distinguish clinical HF not only from healthy controls but also from non-HF forms of dyspnea [40]. They revealed that miR423-5p was most strongly related to the clinical diagnosis of HF and receiver-operator-characteristics curve analysis showed miR423-5p to be a diagnostic predictor of HF, with an area under the curve of 0.91 ($p < 0.001$).

From a diagnostic perspective, Goren et al. tried to evaluate a multimarker approach to HF diagnosis [94]. They measured the levels of 186 miRNAs in the sera of 30 stable chronic systolic HF patients and 30 controls. The differences in miRNA levels between the two groups were characterized, and a score, based on the levels of four specific miRNAs with the most significant increase in the HF group (miR-423-5p, miR-320a, miR-22, and miR-92b) was defined. Interestingly, the score was utilized to discriminate HF patients from controls with a sensitivity and specificity of 90 %. Moreover, in the HF group, there was a significant association between the score and important clinical parameters such as elevated serum natriuretic peptide levels, a wide QRS, and dilatation of the left ventricle and left atrium. These results suggested that a multimarker approach is useful for the detection of not only HF but also left ventricular structure and function.

miRNAs are also related to a more specific cause of HF, such as chemotherapy-induced HF or obesity-related HF. It has been proposed that miRNAs can exert their roles in response to treatment with chemotherapeutic agents. For example, it was suggested that upregulation of miR-146a after doxorubicin (Dox) treatment is involved in acute Dox-induced cardiotoxicity by targeting ErbB4 [95]. Inhibition of both ErbB2 and ErbB4 signaling may be one of the reasons why those patients who receive concurrent therapy with Dox and trastuzumab suffer from HF.

miRNA microarray analyses and real-time polymerase chain reaction have revealed that miR-451 levels were significantly increased in type 2 diabetes mellitus mouse hearts [96]. Calcium-binding protein 39 (Cab39) is a scaffold protein of liver kinase B1 (LKB1), an upstream kinase of AMP-activated protein kinase (AMPK). Cab39 is a direct target of miR-451 in neonatal rat cardiac myocytes, and Cab39 overexpression rescued lipotoxicity. Protein levels of Cab39 and phosphorylated AMPK were increased and phosphorylated mammalian target of rapamycin was reduced in cardiomyocyte-specific miR-451 knockout mouse hearts compared with control mouse hearts. Thus, these results demonstrated that miR-451 is involved in diabetic cardiomyopathy through suppression of the LKB1/AMPK pathway.

Conclusion

miRNAs have emerged as powerful and dynamic modifiers of cardiovascular diseases. The miRNA species discussed above are able to directly regulate the expression of transcription factors, signaling molecules, contractile proteins, and play critical roles in cardiovascular remodeling. Work from several investigators have demonstrated the ability of exogenously administered miRNA inhibitors or miRNA mimics to modulate these pathological processes, thereby ameliorating cardiovascular diseases, which is promising and potentially opens the door for novel therapeutic approaches in the future. The potential of circulating miRNAs as biomarkers for cardiovascular diseases is also in its early stages. Their roles as prognostic biomarkers have yet to be elucidated, and larger studies with longer follow-up periods will be needed.

References

1. Kairouz V, Lipskaia L, Hajjar RJ, Chemaly ER. Molecular targets in heart failure gene therapy: current controversies and translational perspectives. Ann N Y Acad Sci. 2012;1254:42–50.
2. Olson EN. Gene regulatory networks in the evolution and development of the heart. Science. 2006;313:1922–7.
3. Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H. Return to the fetal gene program protects the stressed heart: a strong hypothesis. Heart Fail Rev. 2007;12:331–43.
4. Nakao K, Minobe W, Roden R, Bristow MR, Leinwand LA. Myosin heavy chain gene expression in human heart failure. J Clin Invest. 1997;100:2362–70.
5. Miyata S, Minobe W, Bristow MR, Leinwand LA. Myosin heavy chain isoform expression in the failing and nonfailing human heart. Circ Res. 2000;86:386–90.
6. Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, Doevendans PA, Mummery CL, Borlak J, Haverich A, Gross C, Engelhardt S, Ertl G, Bauersachs J. MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. Circulation. 2007;116:258–67.

7. van Rooij E, Sutherland LB, Liu N, Williams AH, McAnally J, Gerard RD, Richardson JA, Olson EN. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci U S A. 2006;103:18255–60.
8. Chen JF, Murchison EP, Tang R, Callis TE, Tatsuguchi M, Deng Z, Rojas M, Hammond SM, Schneider MD, Selzman CH, Meissner G, Patterson C, Hannon GJ, Wang DZ. Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. Proc Natl Acad Sci U S A. 2008;105:2111–6.
9. Cheng Y, Ji R, Yue J, Yang J, Liu X, Chen H, Dean DB, Zhang C. MicroRNAs are aberrantly expressed in hypertrophic heart: do they play a role in cardiac hypertrophy? Am J Pathol. 2007;170:1831–40.
10. Sayed D, Hong C, Chen IY, Lypowy J, Abdellatif M. MicroRNAs play an essential role in the development of cardiac hypertrophy. Circ Res. 2007;100:416–24.
11. Ikeda S, Kong SW, Lu J, Bisping E, Zhang H, Allen PD, Golub TR, Pieske B, Pu WT. Altered microRNA expression in human heart disease. Physiol Genomics. 2007;31:367–73.
12. Tatsuguchi M, Seok HY, Callis TE, Thomson JM, Chen JF, Newman M, Rojas M, Hammond SM, Wang DZ. Expression of microRNAs is dynamically regulated during cardiomyocyte hypertrophy. J Mol Cell Cardiol. 2007;42:1137–41.
13. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. Curr Biol. 2002;12:735–9.
14. van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. Science. 2007;316:575–9.
15. Callis TE, Pandya K, Seok HY, Tang RH, Tatsuguchi M, Huang ZP, Chen JF, Deng Z, Gunn B, Shumate J, Willis MS, Selzman CH, Wang DZ. MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. J Clin Invest. 2009;119:2772–86.
16. Nishi H, Ono K, Horie T, Nagao K, Kinoshita M, Kuwabara Y, Watanabe S, Takaya T, Tamaki Y, Takanabe-Mori R, Wada H, Hasegawa K, Iwanaga Y, Kawamura T, Kita T, Kimura T. MicroRNA-27a regulates beta cardiac myosin heavy chain gene expression by targeting thyroid hormone receptor {beta}1 in neonatal rat ventricular myocytes. Mol Cell Biol. 2011;31:744–55.
17. van Rooij E, Quiat D, Johnson BA, Sutherland LB, Qi X, Richardson JA, Kelm Jr RJ, Olson EN. A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. Dev Cell. 2009;17:662–73.
18. Bell ML, Buvoli M, Leinwand LA. Uncoupling of expression of an intronic microRNA and its myosin host gene by exon skipping. Mol Cell Biol. 2010;30:1937–45.
19. Li Q, Song XW, Zou J, Wang GK, Kremneva E, Li XQ, Zhu N, Sun T, Lappalainen P, Yuan WJ, Qin YW, Jing Q. Attenuation of microRNA-1 derepresses the cytoskeleton regulatory protein twinfilin-1 to provoke cardiac hypertrophy. J Cell Sci. 2010;123:2444–52.
20. Elia L, Contu R, Quintavalle M, Varrone F, Chimenti C, Russo MA, Cimino V, De Marinis L, Frustaci A, Catalucci D, Condorelli G. Reciprocal regulation of microRNA-1 and insulin-like growth factor-1 signal transduction cascade in cardiac and skeletal muscle in physiological and pathological conditions. Circulation. 2009;120:2377–85.
21. Ikeda S, He A, Kong SW, Lu J, Bejar R, Bodyak N, Lee KH, Ma Q, Kang PM, Golub TR, Pu WT. MicroRNA-1 negatively regulates expression of the hypertrophy-associated calmodulin and Mef2a genes. Mol Cell Biol. 2009;29:2193–204.
22. Kumarswamy R, Lyon AR, Volkmann I, Mills AM, Bretthauer J, Pahuja A, Geers-Knorr C, Kraft T, Hajjar RJ, Macleod KT, Harding SE, Thum T. SERCA2a gene therapy restores microRNA-1 expression in heart failure via an Akt/FoxO3A-dependent pathway. Eur Heart J. 2012;33:1067–75.
23. Care A, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, Bang ML, Segnalini P, Gu Y, Dalton ND, Elia L, Latronico MV, Hoydal M, Autore C, Russo MA, Dorn 2nd GW, Ellingsen O, Ruiz-Lozano P, Peterson KL, Croce CM, Peschle C, Condorelli G. MicroRNA-133 controls cardiac hypertrophy. Nat Med. 2007;13:613–8.

24. Matkovich SJ, Wang W, Tu Y, Eschenbacher WH, Dorn LE, Condorelli G, Diwan A, Nerbonne JM, Dorn 2nd GW. MicroRNA-133a protects against myocardial fibrosis and modulates electrical repolarization without affecting hypertrophy in pressure-overloaded adult hearts. Circ Res. 2010;106:166–75.
25. Ren XP, Wu J, Wang X, Sartor MA, Qian J, Jones K, Nicolaou P, Pritchard TJ, Fan GC. MicroRNA-320 is involved in the regulation of cardiac ischemia/reperfusion injury by targeting heat-shock protein 20. Circulation. 2009;119:2357–66.
26. Hermeking H. p53 enters the microRNA world. Cancer Cell. 2007;12:414–8.
27. Bernardo BC, Gao XM, Winbanks CE, Boey EJ, Tham YK, Kiriazis H, Gregorevic P, Obad S, Kauppinen S, Du XJ, Lin RC, McMullen JR. Therapeutic inhibition of the miR-34 family attenuates pathological cardiac remodeling and improves heart function. Proc Natl Acad Sci U S A. 2012;109:17615–20.
28. Boon RA, Iekushi K, Lechner S, Seeger T, Fischer A, Heydt S, Kaluza D, Treguer K, Carmona G, Bonauer A, Horrevoets AJ, Didier N, Girmatsion Z, Biliczki P, Ehrlich JR, Katus HA, Muller OJ, Potente M, Zeiher AM, Hermeking H, Dimmeler S. MicroRNA-34a regulates cardiac ageing and function. Nature. 2013;495:107–10.
29. Dong S, Cheng Y, Yang J, Li J, Liu X, Wang X, Wang D, Krall TJ, Delphin ES, Zhang C. MicroRNA expression signature and the role of microRNA-21 in the early phase of acute myocardial infarction. J Biol Chem. 2009;284(43):29514–25.
30. Qian L, Van Laake LW, Huang Y, Liu S, Wendland MF, Srivastava D. miR-24 inhibits apoptosis and represses Bim in mouse cardiomyocytes. J Exp Med. 2011;208:549–60.
31. Hu S, Huang M, Nguyen PK, Gong Y, Li Z, Jia F, Lan F, Liu J, Nag D, Robbins RC, Wu JC. Novel microRNA prosurvival cocktail for improving engraftment and function of cardiac progenitor cell transplantation. Circulation. 2011;124:S27–34.
32. Wang JX, Jiao JQ, Li Q, Long B, Wang K, Liu JP, Li YR, Li PF. miR-499 regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1. Nat Med. 2011;17:71–8.
33. Li J, Donath S, Li Y, Qin D, Prabhakar BS, Li P. miR-30 regulates mitochondrial fission through targeting p53 and the dynamin-related protein-1 pathway. PLoS Genet. 2010;6, e1000795.
34. Aurora AB, Mahmoud AI, Luo X, Johnson BA, van Rooij E, Matsuzaki S, Humphries KM, Hill JA, Bassel-Duby R, Sadek HA, Olson EN. MicroRNA-214 protects the mouse heart from ischemic injury by controlling Ca(2)(+) overload and cell death. J Clin Invest. 2012;122:1222–32.
35. Cha MJ, Jang JK, Ham O, Song BW, Lee SY, Lee CY, Park JH, Lee J, Seo HH, Choi E, Jeon WM, Hwang HJ, Shin HT, Hwang KC. MicroRNA-145 suppresses ROS-induced Ca2+ overload of cardiomyocytes by targeting CaMKIIdelta. Biochem Biophys Res Commun. 2013;435:720–6.
36. Li R, Yan G, Li Q, Sun H, Hu Y, Sun J, Xu B. MicroRNA-145 protects cardiomyocytes against hydrogen peroxide (H(2)O(2))-induced apoptosis through targeting the mitochondria apoptotic pathway. PLoS One. 2012;7, e44907.
37. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9:654–9.
38. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, Xiao T, Schafer J, Lee ML, Schmittgen TD, Nana-Sinkam SP, Jarjoura D, Marsh CB. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One. 2008;3, e3694.
39. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105:10513–8.
40. Tijsen AJ, Creemers EE, Moerland PD, de Windt LJ, van der Wal AC, Kok WE, Pinto YM. MiR423-5p as a circulating biomarker for heart failure. Circ Res. 2010;106:1035–9.

41. Wang GK, Zhu JQ, Zhang JT, Li Q, Li Y, He J, Qin YW, Jing Q. Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. Eur Heart J. 2010;31:659–66.
42. Kuwabara Y, Ono K, Horie T, Nishi H, Nagao K, Kinoshita M, Watanabe S, Baba O, Kojima Y, Shizuta S, Imai M, Tamura T, Kita T, Kimura T. Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage. Circ Cardiovasc Genet. 2011;4:446–54.
43. Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, Weber M, Hamm CW, Roxe T, Muller-Ardogan M, Bonauer A, Zeiher AM, Dimmeler S. Circulating microRNAs in patients with coronary artery disease. Circ Res. 2010;107:677–84.
44. Rossi MA. Pathologic fibrosis and connective tissue matrix in left ventricular hypertrophy due to chronic arterial hypertension in humans. J Hypertens. 1998;16:1031–41.
45. Swynghedauw B. Molecular mechanisms of myocardial remodeling. Physiol Rev. 1999; 79:215–62.
46. Manabe I, Shindo T, Nagai R. Gene expression in fibroblasts and fibrosis: involvement in cardiac hypertrophy. Circ Res. 2002;91:1103–13.
47. Brown RD, Ambler SK, Mitchell MD, Long CS. The cardiac fibroblast: therapeutic target in myocardial remodeling and failure. Annu Rev Pharmacol Toxicol. 2005;45:657–87.
48. Khan R, Sheppard R. Fibrosis in heart disease: understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease and arrhythmia. Immunology. 2006;118:10–24.
49. Martos R, Baugh J, Ledwidge M, O'Loughlin C, Conlon C, Patle A, Donnelly SC, McDonald K. Diastolic heart failure: evidence of increased myocardial collagen turnover linked to diastolic dysfunction. Circulation. 2007;115:888–95.
50. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA, Olson EN. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci U S A. 2008;105:13027–32.
51. Ichimura A. miRNAs and regulation of cell signaling. FEBS J. 2011;278(10):1610–8.
52. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, Just S, Rottbauer W, Frantz S, Castoldi M, Soutschek J, Koteliansky V, Rosenwald A, Basson MA, Licht JD, Pena JT, Rouhanifard SH, Muckenthaler MU, Tuschl T, Martin GR, Bauersachs J, Engelhardt S. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature. 2008;456:980–4.
53. Bronnum H, Andersen DC, Schneider M, Sandberg MB, Eskildsen T, Nielsen SB, Kalluri R, Sheikh SP. miR-21 promotes fibrogenic epithelial-to-mesenchymal transition of epicardial mesothelial cells involving programmed cell death 4 and sprouty-1. PLoS One. 2013;8, e56280.
54. Roy S, Khanna S, Hussain SR, Biswas S, Azad A, Rink C, Gnyawali S, Shilo S, Nuovo GJ, Sen CK. MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovasc Res. 2009;82:21–9.
55. Park MJ, Kim MS, Park IC, Kang HS, Yoo H, Park SH, Rhee CH, Hong SI, Lee SH. PTEN suppresses hyaluronic acid-induced matrix metalloproteinase-9 expression in U87MG glioblastoma cells through focal adhesion kinase dephosphorylation. Cancer Res. 2002;62:6318–22.
56. Zheng H, Takahashi H, Murai Y, Cui Z, Nomoto K, Niwa H, Tsuneyama K, Takano Y. Expressions of MMP-2, MMP-9 and VEGF are closely linked to growth, invasion, metastasis and angiogenesis of gastric carcinoma. Anticancer Res. 2006;26:3579–83.
57. Duisters RF, Tijsen AJ, Schroen B, Leenders JJ, Lentink V, van der Made I, Herias V, van Leeuwen RE, Schellings MW, Barenbrug P, Maessen JG, Heymans S, Pinto YM, Creemers EE. miR-133 and miR-30 regulate connective tissue growth factor: implications for a role of microRNAs in myocardial matrix remodeling. Circ Res. 2009;104:170–8. 6p following 8.

58. Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ, Srivastava D. Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell. 2007;129:303–17.
59. Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, Zhang Y, Xu C, Bai Y, Wang H, Chen G, Wang Z. The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat Med. 2007;13:486–91.
60. Jongsma HJ. Diversity of gap junctional proteins: does it play a role in cardiac excitation? J Cardiovasc Electrophysiol. 2000;11:228–30.
61. Saffitz JE, Laing JG, Yamada KA. Connexin expression and turnover: implications for cardiac excitability. Circ Res. 2000;86:723–8.
62. Lerner DL, Yamada KA, Schuessler RB, Saffitz JE. Accelerated onset and increased incidence of ventricular arrhythmias induced by ischemia in Cx43-deficient mice. Circulation. 2000;101:547–52.
63. Diaz RJ, Zobel C, Cho HC, Batthish M, Hinek A, Backx PH, Wilson GJ. Selective inhibition of inward rectifier K+ channels (Kir2.1 or Kir2.2) abolishes protection by ischemic preconditioning in rabbit ventricular cardiomyocytes. Circ Res. 2004;95:325–32.
64. Wang Z, Yue L, White M, Pelletier G, Nattel S. Differential distribution of inward rectifier potassium channel transcripts in human atrium versus ventricle. Circulation. 1998;98:2422–8.
65. Xiao J, Luo X, Lin H, Zhang Y, Lu Y, Wang N, Yang B, Wang Z. MicroRNA miR-133 represses HERG K+ channel expression contributing to QT prolongation in diabetic hearts. J Biol Chem. 2007;282:12363–7.
66. Luo X, Xiao J, Lin H, Li B, Lu Y, Yang B, Wang Z. Transcriptional activation by stimulating protein 1 and post-transcriptional repression by muscle-specific microRNAs of IKs-encoding genes and potential implications in regional heterogeneity of their expressions. J Cell Physiol. 2007;212:358–67.
67. Goldoni D, Yarham JM, McGahon MK, O'Connor A, Guduric-Fuchs J, Edgar K, McDonald DM, Simpson DA, Collins A. A novel dual-fluorescence strategy for functionally validating microRNA targets in 3′ untranslated regions: regulation of the inward rectifier potassium channel K(ir)2.1 by miR-212. Biochem J. 2012;448:103–13.
68. Lu Y, Zhang Y, Wang N, Pan Z, Gao X, Zhang F, Shan H, Luo X, Bai Y, Sun L, Song W, Xu C, Wang Z, Yang B. MicroRNA-328 contributes to adverse electrical remodeling in atrial fibrillation. Circulation. 2010;122:2378–87.
69. Liu Z, Zhou C, Liu Y, Wang S, Ye P, Miao X, Xia J. The expression levels of plasma micoRNAs in atrial fibrillation patients. PLoS One. 2012;7, e44906.
70. Yang WJ, Yang DD, Na S, Sandusky GE, Zhang Q, Zhao G. Dicer is required for embryonic angiogenesis during mouse development. J Biol Chem. 2005;280:9330–5.
71. Wang S, Olson EN. AngiomiRs—key regulators of angiogenesis. Curr Opin Genet Dev. 2009;19:205–11.
72. Mendell JT. miRiad roles for the miR-17-92 cluster in development and disease. Cell. 2008;133:217–22.
73. Dews M, Homayouni A, Yu D, Murphy D, Sevignani C, Wentzel E, Furth EE, Lee WM, Enders GH, Mendell JT, Thomas-Tikhonenko A. Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. Nat Genet. 2006;38:1060–5.
74. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A, Burchfield J, Fox H, Doebele C, Ohtani K, Chavakis E, Potente M, Tjwa M, Urbich C, Zeiher AM, Dimmeler S. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. Science. 2009;324:1710–3.
75. Nicoli S, Standley C, Walker P, Hurlstone A, Fogarty KE, Lawson ND. MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis. Nature. 2010;464:1196–200.
76. Harris TA, Yamakuchi M, Ferlito M, Mendell JT, Lowenstein CJ. MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. Proc Natl Acad Sci U S A. 2008;105:1516–21.

77. Endo-Takahashi Y, Negishi Y, Nakamura A, Ukai S, Ooaku K, Oda Y, Sugimoto K, Moriyasu F, Takagi N, Suzuki R, Maruyama K, Aramaki Y. Systemic delivery of miR-126 by miRNA-loaded Bubble liposomes for the treatment of hindlimb ischemia. Sci Rep. 2014;4:3883.
78. Kuehbacher A, Urbich C, Zeiher AM, Dimmeler S. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. Circ Res. 2007;101:59–68.
79. Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, Richardson JA, Bassel-Duby R, Olson EN. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell. 2008;15:261–71.
80. Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, Ivey KN, Bruneau BG, Stainier DY, Srivastava D. miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell. 2008;15:272–84.
81. Chen Y, Gorski DH. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. Blood. 2008;111:1217–26.
82. Fasanaro P, D'Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, Capogrossi MC, Martelli F. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. J Biol Chem. 2008;283:15878–83.
83. Lee DY, Deng Z, Wang CH, Yang BB. MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. Proc Natl Acad Sci U S A. 2007;104:20350–5.
84. Hua Z, Lv Q, Ye W, Wong CK, Cai G, Gu D, Ji Y, Zhao C, Wang J, Yang BB, Zhang Y. MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. PLoS One. 2006;1, e116.
85. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A. 2005;102:13944–9.
86. Suarez Y, Fernandez-Hernando C, Pober JS, Sessa WC. Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. Circ Res. 2007;100:1164–73.
87. Poliseno L, Tuccoli A, Mariani L, Evangelista M, Citti L, Woods K, Mercatanti A, Hammond S, Rainaldi G. MicroRNAs modulate the angiogenic properties of HUVECs. Blood. 2006;108:3068–71.
88. Ji R, Cheng Y, Yue J, Yang J, Liu X, Chen H, Dean DB, Zhang C. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. Circ Res. 2007;100:1579–88.
89. Liu X, Cheng Y, Zhang S, Lin Y, Yang J, Zhang C. A necessary role of miR-221 and miR-T2009;104:476–87.
90. Maegdefessel L, Azuma J, Toh R, Merk DR, Deng A, Chin JT, Raaz U, Schoelmerich AM, Raiesdana A, Leeper NJ, McConnell MV, Dalman RL, Spin JM, Tsao PS. Inhibition of microRNA-29b reduces murine abdominal aortic aneurysm development. J Clin Invest. 2012;122:497–506.
91. Merk DR, Chin JT, Dake BA, Maegdefessel L, Miller MO, Kimura N, Tsao PS, Iosef C, Berry GJ, Mohr FW, Spin JM, Alvira CM, Robbins RC, Fischbein MP. miR-29b participates in early aneurysm development in Marfan syndrome. Circ Res. 2012;110:312–24.
92. Maegdefessel L, Azuma J, Toh R, Deng A, Merk DR, Raiesdana A, Leeper NJ, Raaz U, Schoelmerich AM, McConnell MV, Dalman RL, Spin JM, Tsao PS. MicroRNA-21 blocks abdominal aortic aneurysm development and nicotine-augmented expansion. Sci Transl Med. 2012;4:122ra22.
93. Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, Pasquinelli AE. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell. 2005;122:553–63.
94. Goren Y, Kushnir M, Zafrir B, Tabak S, Lewis BS, Amir O. Serum levels of microRNAs in patients with heart failure. Eur J Heart Fail. 2012;14:147–54.
95. Horie T, Ono K, Nishi H, Nagao K, Kinoshita M, Watanabe S, Kuwabara Y, Nakashima Y, Takanabe-Mori R, Nishi E, Hasegawa K, Kita T, Kimura T. Acute doxorubicin cardiotoxicity

is associated with miR-146a-induced inhibition of the neuregulin-ErbB pathway. Cardiovasc Res. 2010;87:656–64.
96. Kuwabara Y, Horie T, Baba O, Watanabe S, Nishiga M, Usami S, Izuhara M, Nakao T, Nishino T, Otsu K, Kita T, Kimura T, Ono K. MicroRNA-451 exacerbates lipotoxicity in cardiac myocytes and high-fat diet-induced cardiac hypertrophy in mice through suppression of the LKB1/AMPK pathway. Circ Res. 2015;116(2):279–88.

Chapter 11
microRNAs in Essential Hypertension and Blood Pressure Regulation

Francine Z. Marques and Fadi J. Charchar

Abstract Unravelling the complete genetic predisposition to high blood pressure (BP) has proven to be challenging. This puzzle and the fact that coding regions of the genome account for less than 2 % of the entire human DNA support the hypothesis that mechanisms besides coding genes are likely to contribute to BP regulation. Non-coding RNAs, especially microRNAs, are emerging as key players of transcription regulation in both health and disease states. They control basic functions in virtually all cell types relevant to the cardiovascular system and, thus, a direct involvement with BP regulation is highly probable. Here we review the literature about microRNAs associated with regulation of BP and hypertension, highlighting investigations, methodology and difficulties arising in the field. These molecules are being studied for exploitation in diagnostics, prognostics and therapeutics in many diseases. There have been some studies that examined biological fluid microRNAs as biomarkers for hypertension, but most remain inconclusive due to the small sample sizes and differences in methodological standardisation. Fewer studies have analysed tissue microRNA levels in vascular smooth muscle cells and the kidney. Others focused on the interaction between single nucleotide polymorphisms and microRNA binding sites. Studies in animals have shown that angiotensin II, high-salt diet and exercise change microRNA levels in hypertension. Treatment of spontaneously hypertensive rats with a miR-22 inhibitor and treatment of hypertensive Schlager BPH/2J mice with a miR-181a mimic decreased their BP. This supports the use of microRNAs as therapeutic targets in hypertension, and

F.Z. Marques
School of Applied and Biomedical Sciences, Faculty of Science and Technology, Federation University Australia, Mount Helen, VIC, Australia

Heart Failure Research Group, Baker IDI Heart and Diabetes Institute, Melbourne, VIC, Australia
e-mail: f.charchar@federation.edu.au

F.J. Charchar (✉)
School of Applied and Biomedical Sciences, Faculty of Science and Technology, Federation University Australia, Mount Helen, VIC, Australia

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_11

future studies should test the use of other microRNAs found in human association studies. In conclusion, there is a clear need of increased pace of human, animal and functional studies to help us understand the multifaceted roles of microRNAs as critical regulators of the development and physiology of BP.

Keywords Blood pressure • Arterial pressure • Essential hypertension • Circulating microRNAs • Biomarkers • Kidney • Endothelial cells • Plasma

Introduction

The human genome was mapped in its near entirety in 2003, and since then there has been many noteworthy discoveries but at the same time many more new challenges have appeared. Unravelling the complete genetic predisposition to high blood pressure (BP) has proven to be one of these challenges, perhaps due to its phenotypic heterogeneity [1], but it is more likely that there are many mysteries that our genome has not yet revealed.

Only up to 2.2 % of inter-individual variance in BP may be explained by common single nucleotide polymorphisms (SNPs) associated with BP/hypertension identified by the largest genome-wide association meta-analysis [2]. This and the fact that coding regions of the genome account for less than 2 % of the entire human DNA [3] support the hypothesis that other mechanisms besides coding genes are likely to contribute to BP regulation.

In the past decade, non-coding RNAs (i.e., RNA which does not code for a protein) have emerged as key players in transcription regulation in both health and disease states. In contrast to coding regions, which are highly conserved between species, non-coding RNAs are usually stage-, tissue- and species-specific [4–6], and contribute to the complexity and variety of the transcriptome in humans.

The most studied type of non-coding RNA in hypertension is microRNAs (miRNAs). They have been described as master gene regulators since they can regulate downstream gene expression by post-transcriptional mechanisms, specifically by binding to the 3′ untranslated region (UTR) of a messenger RNA (mRNA) [7]. This leads to mRNA degradation or repression of translation (summarised in Fig. 11.1) [7]. The regulatory roles of miRNAs binding to promoters and 5′ UTR has also been described, but remains mostly unclear at present [8], and there are no examples in hypertension so far.

Based on a search conducted on 9 April 2015 at http://www.mirbase.org/, a total of 1881 precursors and 2588 mature miRNAs are listed in the human genome [9], while a recent publication identified as many as 3707 novel mature miRNAs arising from 3494 novel precursors in humans [10]. Each miRNA can regulate several hundred genes, thus being able to influence whole pathways [6]. Here we review the literature concerning miRNAs associated with BP and essential hypertension, and we ask the question—do they play a role in BP regulation?

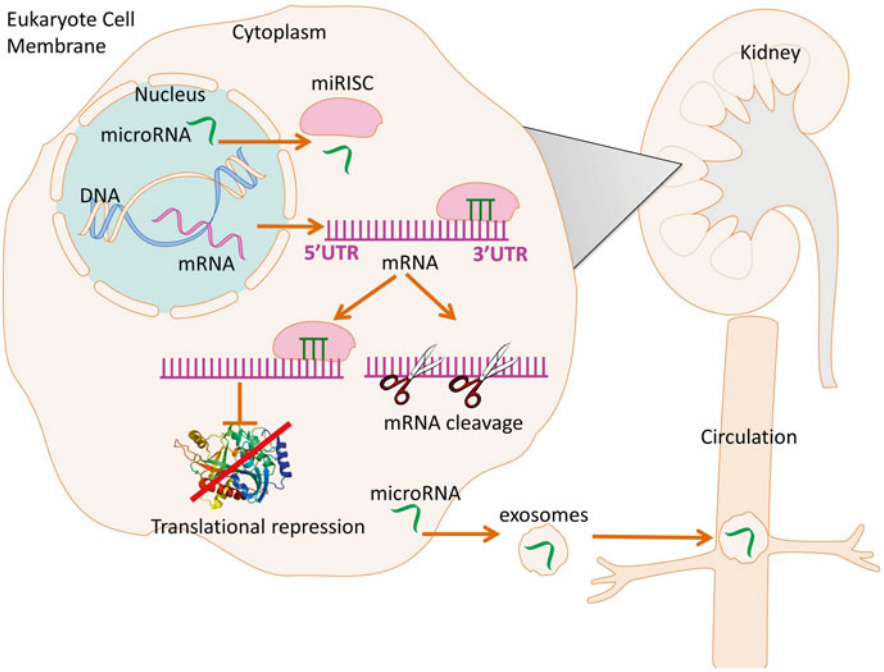

Fig. 11.1 How microRNAs can contribute to blood pressure regulation. Besides the transcription of messenger RNA (mRNA), small non-coding RNAs called microRNAs can also be transcribed in the nucleus of a cell. The mRNA will be exported from the nucleus, and so will be the microRNA, which then binds to the microRNA-mediated silencing complex (miRISC). This complex containing the microRNA then usually binds to the 3′ untranslated region of a mRNA, and as a consequence there is either the translation of repression or the cleavage of the mRNA. This process happens in all tissues of the body, including the kidneys, which can contribute to blood pressure regulation. Moreover, microRNAs can be exported from the tissue of origin in microvesicles, such as exosomes, and, thus, can be detected in the circulation, such as in plasma and serum samples

Methods

The search methodology in this chapter is described in Fig. 11.2. Briefly, we used relevant keywords to search the free-engine PubMed (www.pubmed.com) database of references, and then manually excluded those articles which were not appropriate for inclusion, based on the exclusion criteria described in Fig. 11.1. No article was excluded based on year, and animal and human investigations were included. Some studies have investigated the role of miRNAs in vitro only, and were not included in this chapter. Our search resulted in 38 original articles in miRNAs and hypertension and/or BP, discussed below.

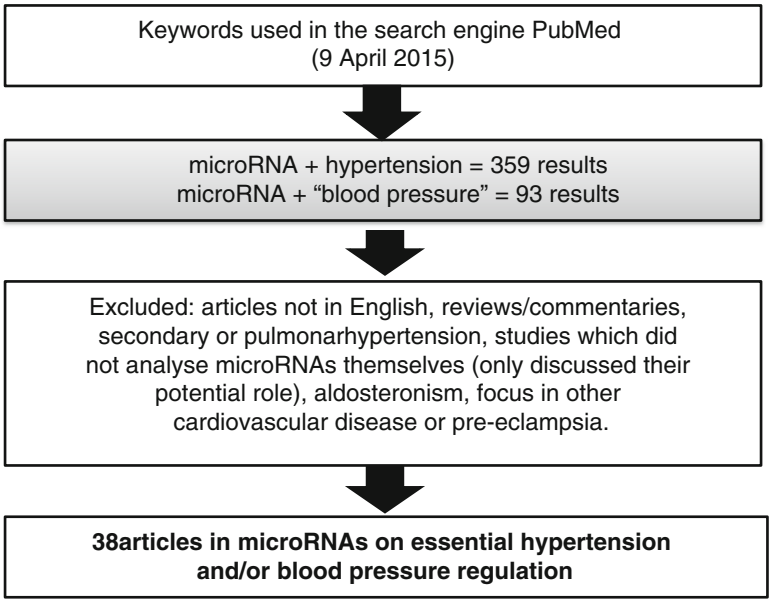

Fig. 11.2 Flow chart of methodology used to select the original investigations included in this chapter. Adapted from [11]

Human Studies

Some of the text below was adapted from reference [11], a review focusing on human non-coding RNAs and hypertension, recently published by the authors. Findings in humans are summarised in Figs. 11.3 and 11.4.

Biological Fluid and Circulating microRNAs

Biological fluid miRNAs are those which are isolated from urine or circulation, such as plasma, serum and blood cells (including vascular endothelial and peripheral blood mononuclear cells). Plasma, serum and urine miRNAs are protected from endogenous RNase-activity because they are carried in extracellular vesicles (such as apoptotic bodies and exosomes), RNA-binding proteins and lipoprotein complexes [12]. These miRNAs are unique in that they are highly stable even when exposed to storage at room temperature or extreme conditions, such as multiple freeze-thaw cycles, boiling and extreme pH [13]. Although body fluids are easily obtainable, measuring circulating levels of miRNAs in them remains challenging.

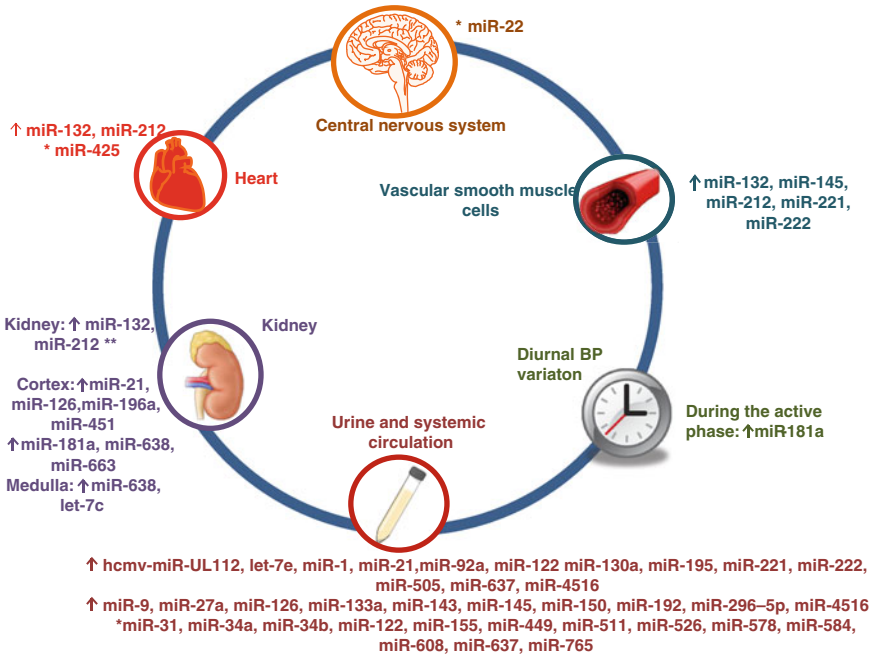

Fig. 11.3 Schematic figure of microRNAs likely to be involved in the regulation of blood pressure and/or predisposition to hypertension, acting in different tissues in the human body. *Down arrows* indicate down-regulated and *up arrows* indicate up-regulated microRNAs, while *asterisks* indicate single nucleotide polymorphisms in binding site of microRNA in BP associated genes. *Double asterisk* indicates no division between renal medulla and cortex was made in the study, so microRNAs were grouped together as 'kidney'. *miR* microRNA. Adapted from [11]

One of the main difficulties in measuring circulating miRNAs is their low concentration (sometimes requiring the need to pool samples), and thus the difficulty in accurately measuring their levels. The most common technique used to measure circulating miRNA concentrations is quantitative real-time polymerase chain reaction (qPCR) using relative quantification. In relative quantification, genes (referred to as housekeeping genes) involved in basic cell maintenance are expected to be expressed in the same tissue at similar levels in health and disease states [14]. The housekeeping genes are used as a reference to the quantity of expression of the gene of interest. In the measurement of circulating miRNAs, there are also technical difficulties due to the lack of accepted standards in normalising the data. There are discussions in favour of exogenous (a RNA transcript specific to other species introduced during the RNA extraction, termed 'spike-in') and endogenous miRNAs or small RNAs (such as RNU6, produced by the cells), or the use of both as normalisers. We refer the reader to a good review on methods used for normalisation during the measurement of miRNAs in body fluids [12].

A genome-wide miRNA study in plasma compared miRNA expression in 13 hypertensive patients and 5 normotensive Chinese subjects by microarray [15].

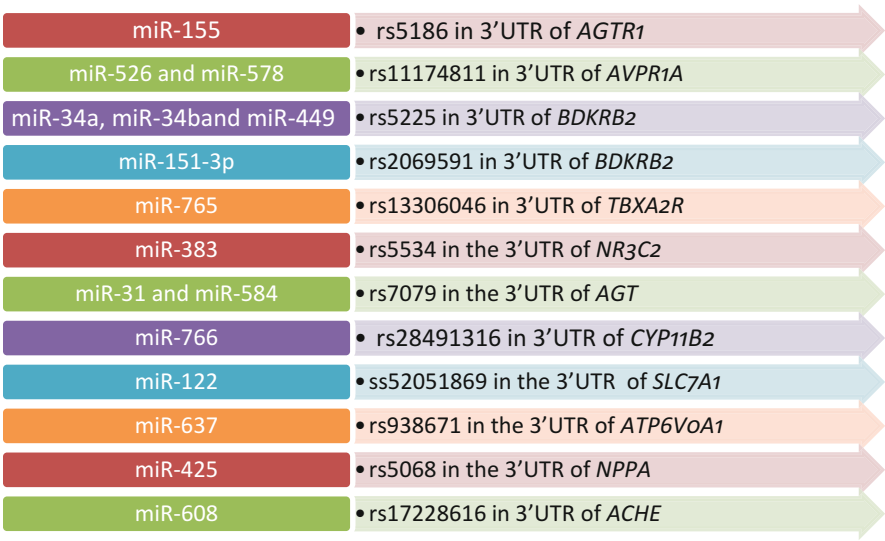

Fig. 11.4 Single nucleotide polymorphisms (SNPs) that create a binding site for a microRNA in blood pressure associated genes

The authors found 27 differentially expressed miRNAs, 9 miRNAs were up-regulated and 18 miRNAs that were down-regulated in hypertensives [15]. They then validated three miRNAs using qPCR—hcmv-miR-UL112, miR-296-5p, and let-7e in a larger cohort of 127 patients and 67 control subjects [15]. The miRNA hcmv-miR-UL112 is a human cytomegalovirus (HCMV)-encoded miRNA. Using in vitro transfection of human embryonic kidney 293 (HEK293) cells with reporter gene constructs, the authors demonstrated that levels for the interferon regulatory factor 1 transcript can be regulated by hcmv-miR-UL112 [15]. The authors also found higher titres of human cytomegalovirus in hypertensives, and hence suggested that cytomegalovirus is responsible for the differential expression of hcmv-miR-UL112 in hypertensives versus normotensives in circulating vascular endothelial cells [15].

The interesting, yet highly speculative, conclusion from this study is that cytomegalovirus could contribute to the elevation in BP rather than be an innocent bystander. Instead, it could be a consequence, in that hypertension-induced pathology could increase susceptibility to cytomegalovirus [16]. Despite the strong correlation between virus titres and blood pressure, a primary association with other conditions more common in hypertension cannot be discounted, especially in the 47 % of non-infected hypertensive patients [16].

In a study aimed to analyse metabolic syndrome and its risk factors, miRNAs were measured in healthy controls compared to those with metabolic syndrome, type 2 diabetes, hypercholesterolemia or hypertension [17]. Consistently with a role of the immune system in hypertension [18], differentially expressed miRNAs were

identified in both blood and exosomes isolated from serum [17]. miR-150, miR-192 and miR-27a were down-regulated in subjects with hypercholesterolemia or hypertension [17]. miRNAs miR-130a, miR-195 and miR-92a were up-regulated in hypertensives and those with the metabolic syndrome [17]. Blood miR-130a and miR-195 were also positively correlated with BP [17]. Only miR-92a, predicted to target the angiotensin II (Ang II) receptor type I (AGTR1) mRNA, was present in exosomes, and its expression reflected the one in blood [17].

This is contradictory to findings in mice deficient in miR-92a, as these mice do not exhibit changes in BP [19]. In agreement with the study by Karoline et al. [17], miR-27a was down-regulated in aortas of the spontaneously hypertensive rat (SHR) compared to Wistar-Kyoto (WKY) [20]. Exercise was able to normalise both miRNA and BP to WKY levels [20].

The expression of urine miRNAs, isolated from exosomes, were investigated in a cohort of ten salt-sensitive, inverse salt-sensitive (i.e., subjects whose BP decreases in response to high salt intake) and salt-resistant subjects (i.e., subjects whose BP increases in response to high salt intake) [21]. These authors reported 45 differentially expressed miRNAs secreted from the kidney in salt-sensitive and salt-resistant individuals or between the inverse salt-sensitive and salt-resistant subjects [21]. The only miRNA that could differentiate the two extremes (salt sensitivity or inverse salt sensitivity) versus the salt-resistant group was hsa-miR-4516. Although the findings have not been replicated in a larger cohort, this study demonstrates that miRNAs possibly derived from kidney tubule cells could be altered or even used as biomarkers for aberrant sodium regulatory pathways in hypertension.

In order to gain insights into molecular mechanisms by which vascular smooth muscle cells (VSMC) affect vascular resistance, Kontaraki and colleagues compared the expression of miR-143, miR-145, miR-21, miR-133 and miR-1 in peripheral blood mononuclear cells between 60 patients with essential hypertension and 29 healthy normotensive subjects [22]. All of the hypertensive patients underwent 24 h ambulatory BP monitoring. The specific miRNAs were chosen based on their previously published role in VSMC phenotype and differentiation. Hypertensive subjects had lower hsa-miR-143, hsa-miR-145 and hsa-miR-133a, and higher hsa-miR-21 and hsa-miR-1. Twenty-four hours mean diastolic BP (DBP) was negative correlated with hsa-miR-143, hsa-miR-145 and hsa-miR-21, whilst mean DBP and hsa-miR-133a were positively correlated [22]. Although these miRNAs were highly expressed in VSMC, the authors did not determine whether these miRNAs were truly derived from VSMC or if they may have originated from other sources. Further investigations are warranted here to determine the role of these miRNAs in inflammation.

Another study from the same group focused on the expression levels of hsa-miR-9 and hsa-miR-126 in peripheral blood mononuclear cells in normotensive compared to hypertensive subjects [23]. Both miRNAs had lower expression in those with high BP, and they were positively correlated with pulse pressure [23]. miR-9 was also positively correlated with left ventricular mass [23]. This supports a role for these miRNAs as markers of advanced target-organ damage in hypertension.

A recent study investigated the expression of hsa-miR-221 and hsa-miR-222 in circulating progenitor cells (also termed pro-angiogenic haematopoietic cells), selected by CD34 surface antigen. They compared cells from normotensive and hypertensive subjects, the latest being subdivided into those with carotid intima-media thickness but without left ventricular hypertrophy, and those with left ventricular hypertrophy but without carotid intima-media thickness [24]. These miRNAs were chosen based on their association with previously reported regulatory roles in angiogenesis, cell proliferation and vascular inflammation. Hypertensive subjects had increased reactive oxygen species (ROS), C-reactive protein (CRP), fibrinogen and a higher number of CD34+ cells. These heightened inflammation parameters were associated with an increase in hsa-miR-221 and hsa-miR-222 expression [24]. In the hypertensive group with left ventricular hypertrophy, the miRNAs were negatively correlated with the number of CD34+ cells and positively correlated with ROS [24]. The higher levels of hsa-miR-221 and hsa-miR-222 are consistent with increased ROS production and inflammatory markers, but a direct involvement with impairment of circulating progenitor cells is yet to be established.

Microarrays were used to investigate the whole-plasma miRNA expression of six healthy controls and six hypertensive Chinese patients, and found three up-regulated miRNAs in hypertension: hsa-miR-425, hsa-miR-505 and hsa-miR-210 [25]. These findings were replicated in two other cohorts (11 healthy, 20 pre-hypertensive and 19 hypertensive subjects, and 91 healthy and 101 hypertensive subjects) [25]. The levels of miR-505 were consistently higher in hypertensives in all three cohorts [25]. This miRNA was found to play a role in angiogenesis by impairing endothelial cell migration and tube formation, through the regulation of the gene for fibroblast growth factor 18 (*FGF18*) [25].

Ten miRNAs were studied in plasma samples of a cohort of 30 normotensive, 30 hypertensive and 30 white-coat hypertensive subjects [26]. The expression of miR-21, miR-122, miR-637 and let-7e were up-regulated in hypertensives compared to normotensives, while miR-122 and miR-637 were higher in white-coat patients than in control subjects [26]. The miRNA miR-296-5p was down-regulated in hypertensives, but up-regulated in white-coat hypertensives [26]. The expression of both miR-296-5p and miR-637 were able to distinguish between hypertensives and white-coat hypertensives [26]. Ambulatory, SBP and DBP were negatively correlated with miR-296-5p [26]. This was the first study to analyse miRNAs in white-coat hypertensive patients, and it highlighted their use to distinguish between subtypes of hypertension.

In summary, there is still little, but growing, evidence for the use of circulating miRNA as biomarkers for hypertension. The field is still in its infancy and this may change over the next few years. Larger sample sizes and improvements in methodology, such as the identification of markers of exosomal membrane that distinguish tissues and different cell types, will be useful. This can be used to pinpoint where the circulating exosomes, and thus miRNAs, are coming from and their potential roles. The study of circulating exosomes would highly improve currently limited knowledge of the interaction between compartments and cell-to-cell signalling. Another limitation is related to the use of qPCR technology: miRNA sequences are

amplified using oligonucleotides specific for small nucleic acids, and the amount of miRNA is very low. The amplification step may lead to artefacts if not properly performed with rigorous controls.

Recent advances such as digital PCR (which can measure a single copy of a miRNA) or the use of small RNA-sequencing (requiring as little as 5 ng as described in [27]) are improving the precision of miRNA measurement [28]. Researchers still need to consider whether preamplification is used or not, what housekeeping miRNAs (if endogenous or exogenous, or preferably both) are chosen, and the number of exosomes and cells collected so as to allow for accurate assessment of the physiological relevance of each miRNA.

Tissue microRNAs

We have previously described genome-wide changes in genes and miRNAs measured by microarrays in the renal cortex and medulla of hypertensive ($n=5$ and $n=9$, respectively) and normotensive ($n=3$ and $n=5$, respectively) European subjects who underwent elective unilateral nephrectomies because of non-invasive renal cancer [29]. This unique cohort, collected specifically to study the molecular aspects of human cardiovascular disease [30, 31], might provide further insights about renal mechanisms which contribute to BP regulation.

The RNA samples were derived from a pole of kidney unaffected by the neoplastic process. In the renal medulla, 12 genes and 11 miRNAs were differentially expressed, and in the cortex, 46 genes and 13 miRNAs were differentially expressed. The differentially expressed miRNAs and genes were further investigated in 22 hypertensives and 16 normotensives. In the renal medulla samples, we validated by qPCR the miRNAs hsa-miR-638 and hsa-let-7c, and in the cortex samples hsa-miR-21, hsa-miR-126, hsa-miR-181a, hsa-miR-196a, hsa-miR-451, hsa-miR-638 and hsa-miR-663. Two under-expressed miRNAs, hsa-miR-181a and hsa-miR-663, were of particular interest because in silico analyses had shown they could target specific sequences in the human renin 3′ UTR mRNA. Furthermore, by means of co-transfection experiments in vitro using HEK293 cells, we showed that hsa-miR-181a and hsa-miR-663 bind to renin, and also revealed that these miRNAs were able to regulate endogenous renin mRNA levels. These results suggest that a change in these miRNAs could explain the overexpression of renin mRNA observed in the hypertensive kidneys.

Due to the well-recognised role of the renin-angiotensin-aldosterone system (RAAS) in BP regulation, the mechanisms of action of Ang II involving miRNAs have also been investigated in mice, rats and humans. A study by Eskildsen and colleagues examined global miRNA expression in the left ventricle of Sprague-Dawley rats treated with a constant intravenous infusion of Ang II for 10 days that lead to fibrosis and cardiac hypertrophy [32]. In the heart, aorta and kidneys of the Ang II treated rats the authors identified that the cluster composed of the miRNAs miR-132 and miR-212 were up-regulated, and suggested that this may mediate Ang II-induced hypertension [32].

These miRNAs were also overexpressed in rats treated with endothelin-1 for 10 days, but no fibrosis or cardiac hypertrophy was evident at the physiological or molecular level. Moreover the expression of these miRNAs was attenuated in internal mammalian artery (also known as internal thoracic artery, which supplies blood for the anterior chest wall) of patients treated with AGTR1 blockers compared to those treated with β-blockers [32]. Although the authors speculate the involvement of Gαq, in addition to the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), a thorough investigation of miR-132 and miR-212 gene targets is needed to fully elucidate their contribution to BP regulation.

Santovito and colleagues measured the expression of hsa-miR-145, a miRNA that is involved in VSMC proliferation and vascular tone in human atherosclerotic plaques from 22 subjects with and without essential hypertension. The authors found that this miRNA was overexpressed in hypertension, especially in those treated with Ang II receptor blockers [33]. The authors did not investigate the mRNA targets of miR-145, and therefore the genetic mechanisms involved are still to be deciphered.

In summary, there is emerging evidence that miRNAs may be involved in the development of hypertension, yet more evidence is needed. Large tissue banks of human kidneys [30, 31] are now being studied and will play a crucial part in this. In vivo studies treating animal models, such as the SHR, could play an important role to determine whether miRNAs indeed regulate BP. It is clear, however, that there is a need for more research using larger human cohorts and relevant tissues to fully understand the miRNA mechanisms involved in essential hypertension.

SNPS Creating or Modifying microRNA-Binding Sites

SNPs can create or disrupt binding sites for miRNAs. If a miRNA binds preferentially to one allele of an mRNA 3′ UTR than to other allele(s), it results in lower levels of the mRNA or protein. The findings in the hypertension and blood pressure regulation to date are summarised in Fig. 11.4.

One pioneer study in the field of miRNAs and BP described that miRNA hsa-miR-155 could bind more efficiently to the *A* allele than to the *C* allele at position +1166 of the SNP rs5186 in the 3′ UTR of the *AGTR1* mRNA. The *C* allele is also more prevalent in essential hypertension subjects [34]. The binding of hsa-miR-155 will potentially suppress the level of *AGTR1* mRNA and thus the pressor effect of Ang II in subjects with the *A* allele [34].

Further evidence for this is that protein levels of AGTR1 in untreated essential hypertension subjects homozygous for the *C* allele of rs5186 were also positively correlated with systolic (SBP) and DBP. AGTR1 protein levels were also negatively correlated with expression levels of hsa-miR-155, and the miRNA levels were lower in those with the *CC* genotype [35]. These findings are consistent with those in animal models, showing that miR-155 was down-regulated in aortas of the SHR compared to WKY, and exercise was able to normalise miRNA to WKY levels [20].

The modulation of expression of other genes in the RAAS by the preferential binding of miRNAs to SNPs in the 3′ UTR region has been predicted by two other studies [36, 37], of which only one proceeded to experimental validation in vitro [37]. Four out of 10 SNPs within 8 genes in the RAAS were associated with changes in BP. The authors also demonstrated that SNPs in the arginine vasopressin 1A receptor (*AVPR1A*) gene, bradykinin 2 receptor (*BDKRB2*) gene and thromboxane A2 receptor (*TBXA2R*) gene changed the binding site for several miRNAs [37]. Another study showed that the major *G* allele of rs28491316 of the aldosterone synthase (*CYP11B2*) gene creates a binding site for miR-766, thus decreasing *CYP11B2* mRNA and protein levels [38]. Moreover, the miRNAs miR-31 and miR-584 were found to bind preferentially to the major *C* allele of the SNP rs7079 in the angiotensinogen (*AGT*) gene [39]. These miRNAs were also able to down-regulate *AGT* mRNA and protein levels in vitro [39].

Besides the RAAS, other mechanisms commonly known to influence BP have been associated with SNPs that lead to the creation or disruption of miRNA binding sites. The minor *T* allele of the SNP ss52051869 in the 3′ UTR of the gene for L-arginine transporter (*SLC7A1*) was previously associated with endothelial and nitric oxide dysfunction, and increased predisposition to hypertension [40]. The *T* allele of ss52051869 was later associated with the presence of a longer 3′ UTR with more binding sites for the miRNA miR-122 (4 sites vs. 3 sites in the shorter 3′ UTR associated with the *C* allele) [41]. This allele also disrupts the binding of the transcription factor specificity protein 1 (SP1) [41].

Another example is the SNP A+1050G in the 3′ UTR of the neuropeptide Y1 receptor (NPY1R) mRNA, important for adrenergic activity and BP control [42]. Although hsa-miR-511 is predicted to bind preferentially to the *G* allele, this study lacked in vitro co-transfections of miRNA and a plasmid with the specific binding sequence to validate the binding. The presence of the *C* variant of the SNP rs938671 in the 3′ UTR of the gene for vacuolar H^+-ATPase subunit (*ATP6V0A1*) was previously associated with catestatin secretion and BP [43]. This allele was further associated with the down-regulation *ATP6V0A1* mRNA levels [44]. Another study found that having the *C* allele decreased *ATP6V0A1* mRNA and protein levels compared to the *T* allele, and that the miRNA hsa-miR-637 could preferentially bind to the *C* rather than to the *T* allele [44].

The SNP rs5068 in the 3′ UTR of the atrial natriuretic peptide (*NPPA*) gene was previously identified to be associated with essential hypertension and BP in a meta-analysis of genome-wide association studies [2]. Recently, miR-425 was shown to be produced in human atria and ventricles, and to bind to the *A* allele, but not to the *G* allele, of rs5068. This miRNA could also regulate *NPPA* mRNA levels in vitro only when the *A* allele was present [45].

One investigation analysed the impact of the preferential binding of miR-608 to the major *C* allele in comparison to the minor *A* allele of the 3′ UTR SNP rs17228616 in the acetylcholinesterase (*ACHE*) mRNA [46]. Subjects carrying the *A* allele had reduced serum cortisol and higher BP [46]. The *A* allele presented with 65 % higher ACHE hydrolytic activity than the *C* allele [46]. In the presence of the

A allele, and thus less miRNA-mRNA binding, there was a higher availability of miR-608, resulting in the suppression of other mRNA targets of this miRNA [46]. The minor *A* allele was more common in African-Americans (38.4 % carriers) than European-descendants (6.2 % carriers).

A study attempted to find SNPs located within miRNA binding sites in cardiometabolic genes using genome-wide association studies on 17 cardiometabolic diseases [47]. They found two SNPs that had an effect on the host gene expression and were associated with BP: rs7085 in the 3′ UTR of c-src tyrosine kinase (*CSK*) and rs10786736 in the 5′-nucleotidase cytosolic II (*NT5C2*) gene [47]. The miRNAs predicted to bind to these SNPs were not disclosed in the study, and functional experiments were not performed for these loci.

Several of the studies described here lacked in vitro analyses with the overexpression or inhibition of miRNAs and a direct effect on the expression of mRNAs in a specific cell line. All these findings together highlight the importance of the interaction between miRNAs and polymorphisms at the mRNA level, and future studies should explore these interactions at the whole-genome level. Once again the difficulty here will be to acquire relevant tissues, as miRNAs are tissue-specific, and large cohorts would be necessary to ensure sufficient power.

Polymorphisms in microRNA Sequences

Some of the SNPs identified in the genome-wide association meta-analysis for BP and hypertension were not in regions coding [2], and thus could be in regions containing sequences for ncRNAs instead. So far none of these SNPs are in sequences known to contain ncRNAs. Only one study in 156 hypertensives and 187 normotensives analysed whether SNPs in microRNA sequences (miR-143) were associated with essential hypertension [48].

The frequency of the minor *C* allele of the SNP rs4705342 in the promoter of miR-143 was lower in the hypertensive group (39 % controls vs. 27.9 % cases), and it was associated with a lower risk of hypertension than in subjects with the TT genotype [48]. The authors did not investigate whether the presence of this SNP resulted in changes in the expression of miR-143. The genetic association needs to be replicated in a larger, independent cohort.

By the use of the largest genome-wide association studies on 17 cardiometabolic diseases, including the Global BPgen consortium, a study attempted to find genetic variants in seed regions of miRNAs [49]. They found that the SNP rs2168518, in a seed region of miR-4513, was associated with several cardiometabolic phenotypes, including SBP and DBP [49]. The minor *G* allele of this SNP also changed the expression of miR-4513 in vitro [49]. Golgi SNAP receptor complex member 2 (*GOSR2*), associated with SBP, was identified as a target of miR-4513 [49]. We should see an increase in this type of studies in the near future.

Interaction Between Genotypes, Genes and microRNAs

A study investigated whether having the SNPs rs6276, rs6277 and rs1800497 in the dopamine D2 receptor (DRD2) gene resulted in differential expression of miRNAs in human renal proximal tubule cells, isolated from patients who had unilateral nephrectomy due to renal carcinoma or trauma [50].

They found that subjects carrying alternative forms of these SNPs had lower levels of miR-217, miR-224, miR-335 and miR-1265, and higher miR-1290 than those carrying the major alleles [50]. Out of these miRNAs, only miR-217 was directly regulated by the expression of *DRD2* [50]. miR-217, in turn, regulated the levels of transforming growth factor β 1 (*TGFB1*) mRNA and protein and the axis WNT5A-ROR2 (wingless-type MMTV integration site family, member 5A, and receptor tyrosine kinase-like orphan receptor 2) [50].

This study highlights the complex interactions between genotype, miRNAs, expression of genes and pathways leading to hypertension.

Genetic and Environmental Interaction

It is well established that the prevalence of hypertension increases with ageing [51]. Wang and colleagues [52] used a non-linear varying-coefficient model relative to ageing to find genetic markers associated with DBP. They used genome-wide association data of 142 unrelated subjects from the Genetic Analysis Workshop 18 [52]. The region of chromosome 3 which contains the miRNA gene *MIR1263*, including the SNP rs9863717, had a non-linear effect over-time to affect DBP [52]. Carriers of the *GG* genotype for rs9863717 had lower DBP after 55 years of age, while carriers *AA* had higher DBP and chance to develop hypertension [52]. The authors discussed that previous traditional linear models would miss this interaction. Albeit interesting, this needs to be validated in independent and larger cohorts.

Findings in Animal Models

Animal studies are essential to determine the in vivo role of miRNAs in BP regulation and their potential use as therapeutic targets instead of biomarkers only.

The levels of skeletal muscle miRNAs miR-16, miR-21 and miR-126, involved in vascularisation, were investigated to understand how aerobic exercise training lowers BP [53]. Adult SHR and WKY rats swam for 60 min, 5 days a week for 10 weeks [53]. BP was lower in SHR rats that exercised compared to those which did not, while no changes in BP were observed in the WKY strain [53]. Exercise also significantly increased muscle vascularisation and miR-126 in the SHR, and lowered miR-16 and miR-21 [53]. Another study exposed SHR and WKY to exercise training on a treadmill for 12 weeks [20].

The effect of exercise on BP was similar as reported above [20, 53]. They showed that exercise decreased aortic remodelling, and improved endothelial function and the balance between the components of the RAAS [20]. Exercise also increased aortic miR-27a and miR-155, and lowered miR-143, all of which can regulate some components of the RAAS [20]. Together, these studies support the role of miRNAs in lowering BP due to the effect of aerobic exercise in endothelial cells, but this effect seems to be specific to hypertension.

The role of *Dicer*, an enzyme involved in miRNA biogenesis and processing, which cleaves precursor-miRNAs to mature forms, has been investigated in relation to BP. A study generated mice with a conditional deletion of *Dicer* in juxtaglomerular cells, the renal cells that produce renin, the rate-limiting enzyme of the RAAS [54]. This resulted in a reduction in the number of juxtaglomerular cells, >80 % lower renin expression (both *Ren1* and *Ren2* mRNA) and plasma renin concentration, decreasing BP by 15 mmHg and damaging renal function with increased fibrosis [54]. This supports the importance of *Dicer* and miRNAs in the maintenance of juxtaglomerular cells and overall renal function.

Mice lacking both miR-143 and miR-145 had thinner arteries with a reduction in width of smooth muscle differentiation, reduced vascular tone, and thus, significantly lower BP [55]. Another study deleted *Dicer* in smooth muscle cells, which resulted in a global loss of miRNAs in arteries [56]. Contractile function of resistance arteries and aortic medial thickness were reduced [56]. Knockout mice had lower SBP and DBP [56], and the drop in BP was more pronounced than in the miR-143/miR-145 knockout [55], suggesting that other miRNAs are important for VSMC function and BP regulation.

Consistent with the down-regulation of miR-181a in human kidneys [29], the Schlager BHP/2J mouse, a neurogenic model of hypertension with marked circadian elevation of BP [57], has lower levels of miR-181a and higher renin mRNA during the active period [58]. The hypertensive strain also has higher tyrosine hydroxylase staining in the cortical tubules, indicating increased renal sympathetic innervation [58]. When we treated BPH/2J mice with a miR-181a mimic, it decreased BP and renal renin mRNA, supporting a role of this miRNA in the regulation of both BP and renin in hypertension [59].

A recombinant adenoviral vector containing an artificial miRNA designed to target *Agtr1* mRNA was injected in the paraventricular nucleus of SHR and control Wistar rats [60]. This reduced *Agtr1* mRNA in both strains, but a decrease in BP was only observed in the SHR [60]. Plasma norepinephrine, depressor response, plasma Ang II and renal sympathetic nerve activity were also normalised in the SHR, while no change was observed in treated Wistar rats [60].

The miRNA miR-487b was the most differentially regulated miRNA in adult Sprague-Dawley rats with Ang II-induced hypertension [61]. This miRNA was shown to target and co-localised in rat aortic tissue with the gene for insulin receptor substrate 1 (*Irs1*), which has a role in cell proliferation [61]. When miR-487b was inhibited in human umbilical arterial adventitial fibroblast and smooth muscle cell lines, there was a threefold increase in cell survival [61], proposing a mechanism for vascular pathology in hypertension.

Consomic SS-13^{BN} rats, which have the chromosome 13 from the normotensive Brown Norway rat strain in the Dahl salt-sensitive background, had lower BP when exposed to a high-salt diet [62]. A study investigated changes in renal medulla whole-genome miRNA due to a high-salt diet in Dahl salt-sensitive and SS-13^{BN} rats [63]. Two miRNAs (miR-29b and miR-302d) were lower in Dahl salt-sensitive rats, while three were higher (miR365, miR-211 and miR-15b) [63]. miR-29b was predicted to regulate 20 collagen and several extracellular matrix related genes, and the change in expression of several collagen genes was validated in vivo [63]. Treatment with an anti-miR-29b, however, did not change BP [63]. These findings are relevant to the interstitial fibrosis in the medulla as a consequence of high-salt diet.

Another study treated Sprague-Dawley rats with Ang II for 4 weeks, resulting in higher BP and myocardial fibrosis [64]. Moreover, myocardial miR-29b and miR-133 were decreased, and collagen type I α 1 (*Col1a1*) mRNA was increased [64]. This study supports the role of these miRNAs in fibrosis in the presence of Ang II-induced hypertension.

Changes in aortic whole-genome miRNAs were studied in Dahl salt-sensitive rats exposed to a high-salt diet [65]. This resulted in the dysregulation of 37 miRNAs, including the up-regulation of miR-320 and down-regulation of miR-26b and miR-21 [65]. These miRNAs were shown to target insulin growth factor-1 receptor (*Igf1r*) and phosphatase and tensin homolog (*Pten*) mRNA [65]. VSMCs treated with a miR-320 inhibitor or a miR-26b mimic reduced the expression of collagen genes and prevented cells from hypertrophy [65]. The authors then tested whether β-blockers would improve the salt-induced hypertension phenotype by modulating these miRNAs and target genes [65]. While nebivolol, a selective β1 inhibitor with β3 activation function, normalised all three miRNAs, atenolol, a pure β1-selective blocker, normalised miR-26b and increased miR-21 [65]. This investigation supports the use of β-blockers as treatment for salt-induced hypertension through action on miRNAs involved in arterial dysfunction and remodelling.

An example of therapeutic use of a miRNA in hypertension is miR-22, which targets chromogranin A (*Chga*) mRNA [66]. SHR which were treated with four doses of miR-22 inhibitor at 25 mg/kg exhibited a decrease in BP of 18 mmHg in 9 days [66]. Polymorphisms in the gene for *CHGA* and plasma concentrations of CHGA have been associated with human hypertension [67]. A polymorphism in the 3′ UTR, which increases the binding of miR-22, was identified in the SHR [66].

In summary, the therapeutic use of miRNAs is currently being explored through the approaches of overexpression and inhibition in many diseases, including cardiovascular diseases. In hypertension, the only examples are miR-22 and miR-181a, as discussed above. While there have been few studies to date that have assessed the use of miRNAs for anti-hypertensive therapy, there are several examples in other cardiovascular diseases. For example, inhibition of miR-25 in an established model of heart failure improved cardiac contractility and function [68].

Challenges of Studying microRNAs in BP

Essential hypertension is a complex heritable trait thought to involve multiple, mostly unknown, genes, the majority having a small effect and a few genes with large effect, that interact with environmental factors [69]. The study of twins during the past few decades has been used to estimate the heritability of BP, with heritability of SBP varying from 44 to 71 %, and variability of DBP varying from 46 to 74 % [70–75].

A variety of environmental factors are known to contribute to the increased BP in essential hypertension patients. Early life factors, such as dietary sodium intake, childhood exercise, breast feeding and birth weight, contribute to the development of hypertension later in life [76]. Lifestyle factors, such as moderate physical exercise, healthy diet, limitation of cigarette, alcohol and sodium consumption, stress management and maintaining a lean body weight, are known to help in the management of hypertension [77]. Currently, the miRNA literature in hypertension lacks the analysis of environmental factors influencing miRNA expression. Similarly, no studies have analysed the levels of miRNAs according to genotypes and BP. These studies are challenging due to high interpersonal variability, thus requiring large cohorts with detailed characterisation and long follow-up.

Changes in expression of any miRNA, as well as in mRNAs, could either contribute to or be a secondary effect of hypertension. When the sequence of the miRNA is conserved between humans and rodents, the use of animal models might assist in providing physiological support for a putative causative mechanism inferred from molecular and in vitro findings. In humans, besides kidneys and arteries (including VSMC), no other tissues are currently available, limiting advances in the field. The availability of tissue engineering may be of use in that regard in the future [78].

There are several algorithms and tools to predict the interaction between miRNA with protein-coding mRNAs and DNA, but these predictions need validation by time-consuming in vitro techniques, such as luciferase assays for miRNAs and mRNAs. Although well studied in other areas of human disease, deciphering interactions between RNA–RNA and RNA–DNA has been under-researched in BP regulation.

Such studies can, moreover, be challenging, since the effects of miRNAs may only take place under certain conditions, such as certain stressors or specific developmental stages, and might further complicate the findings. Interaction between miRNAs are also possible, such as the role of circular RNAs acting as efficient miRNAs sponges and having several sites for binding of a specific miRNA, thus counteracting their actions [79]. miRNAs have been found to bind to and regulate long intergenic non-coding RNA levels in a similar way as they regulate mRNAs [80].

Conclusions

The recent advent of whole-genome sequencing and (small) RNA sequencing technologies has made more information about the human genome and transcriptome available. They have also revealed the incredibly complex and malleable nature of integrations that exist between gene, RNA and protein. miRNAs are, however, a relatively underdeveloped area of investigation in hypertension research.

Advances have lagged behind those made in other fields such as cancer and heart failure. Research into the individual role of miRNAs and the interaction between different types of miRNAs, DNA polymorphisms and environment is needed in hypertension. Studies using larger cohorts and tissues besides biological fluids should assist in a better understanding of the multifaceted roles of miRNAs. Considering many studies have revealed that miRNAs are critical regulators of cardiovascular development [81] and ageing [82], there are still important opportunities in the field to further understand how they affect hypertension.

Acknowledgements We would like to thank Mr. Ian Goodbody for proofreading this chapter. F.Z.M. and F.J.C. are supported by grants from the National Health & Medical Research Council of Australia (NHMRC), the National Heart Foundation and the Federation University Australia 'Self-sustaining Regions Research and Innovation Initiative', an Australian Government Collaborative Research Network (CRN). F.Z.M. is supported by NHMRC (APP1052659) and National Heart Foundation (PF12M6785) co-shared Early Career Fellowships.

References

1. Munroe PB, Barnes MR, Caulfield MJ. Advances in blood pressure genomics. Circ Res. 2013;112(10):1365–79.
2. Ehret GB, Munroe PB, Rice KM, Bochud M, Johnson AD, Chasman DI, et al. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. Nature. 2011;478(7367):103–9.
3. Mattick JS. Non-coding RNAs: the architects of eukaryotic complexity. EMBO Rep. 2001;2(11):986–91.
4. Frith MC, Pheasant M, Mattick JS. The amazing complexity of the human transcriptome. Eur J Hum Genet. 2005;13(8):894–7.
5. Thomas JW, Touchman JW, Blakesley RW, Bouffard GG, Beckstrom-Sternberg SM, Margulies EH, et al. Comparative analyses of multi-species sequences from targeted genomic regions. Nature. 2003;424(6950):788–93.
6. Esteller M. Non-coding RNAs in human disease. Nat Rev Genet. 2011;12(12):861–74.
7. Pasquinelli AE. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet. 2012;13(4):271–82.
8. Da Sacco L, Masotti A. Recent insights and novel bioinformatics tools to understand the role of microRNAs binding to 5' untranslated region. Int J Mol Sci. 2012;14(1):480–95.
9. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 2014;42(Database issue):D68–73.
10. Londin E, Loher P, Telonis AG, Quann K, Clark P, Jing Y, et al. Analysis of 13 cell types reveals evidence for the expression of numerous novel primate- and tissue-specific microRNAs. Proc Natl Acad Sci U S A. 2015;112(10):E1106–15.

11. Marques FZ, Booth SA, Charchar FJ. The emerging role of non-coding RNA in essential hypertension and blood pressure regulation. J Hum Hypertens. 2015;29(8):459–67.
12. Zampetaki A, Willeit P, Drozdov I, Kiechl S, Mayr M. Profiling of circulating microRNAs: from single biomarkers to re-wired networks. Cardiovasc Res. 2012;93(4):555–62.
13. Tijsen AJ, Pinto YM, Creemers EE. Circulating microRNAs as diagnostic biomarkers for cardiovascular diseases. Am J Physiol Heart Circ Physiol. 2012;303(9):H1085–95.
14. Eisenberg E, Levanon EY. Human housekeeping genes, revisited. Trends Genet. 2013;29(10):569–74.
15. Li S, Zhu J, Zhang W, Chen Y, Zhang K, Popescu LM, et al. Signature microRNA expression profile of essential hypertension and its novel link to human cytomegalovirus infection. Circulation. 2011;124(2):175–84.
16. Marques FZ, Morris BJ. Letter by Marques and Morris regarding article, "Signature microRNA expression profile of essential hypertension and its novel link to human cytomegalovirus infection". Circulation. 2012;125(5):e337. author reply e8-9.
17. Karolina DS, Tavintharan S, Armugam A, Sepramaniam S, Pek SL, Wong MT, et al. Circulating miRNA profiles in patients with metabolic syndrome. J Clin Endocrinol Metab. 2012;97(12):E2271–6.
18. Schiffrin EL. Immune mechanisms in hypertension and vascular injury. Clin Sci (Lond). 2014;126(4):267–74.
19. Penzkofer D, Bonauer A, Fischer A, Tups A, Brandes RP, Zeiher AM, et al. Phenotypic characterization of miR-92a-/- mice reveals an important function of miR-92a in skeletal development. PLoS One. 2014;9(6), e101153.
20. Gu Q, Wang B, Zhang XF, Ma YP, Liu JD, Wang XZ. Contribution of renin-angiotensin system to exercise-induced attenuation of aortic remodeling and improvement of endothelial function in spontaneously hypertensive rats. Cardiovasc Pathol. 2014;23(5):298–305.
21. Gildea JJ, Carlson JM, Schoeffel CD, Carey RM, Felder RA. Urinary exosome miRNome analysis and its applications to salt sensitivity of blood pressure. Clin Biochem. 2013;46(12): 1131–4.
22. Kontaraki JE, Marketou ME, Zacharis EA, Parthenakis FI, Vardas PE. Differential expression of vascular smooth muscle-modulating microRNAs in human peripheral blood mononuclear cells: novel targets in essential hypertension. J Hum Hypertens. 2014;28(8):510–6.
23. Kontaraki JE, Marketou ME, Zacharis EA, Parthenakis FI, Vardas PE. MicroRNA-9 and microRNA-126 expression levels in patients with essential hypertension: potential markers of target-organ damage. J Am Soc Hypertens. 2014;8(6):368–75.
24. Mandraffino G, Imbalzano E, Sardo MA, D'Ascola A, Mamone F, Lo Gullo A, et al. Circulating progenitor cells in hypertensive patients with different degrees of cardiovascular involvement. J Hum Hypertens. 2014;28(9):543–50.
25. Yang Q, Jia C, Wang P, Xiong M, Cui J, Li L, et al. MicroRNA-505 identified from patients with essential hypertension impairs endothelial cell migration and tube formation. Int J Cardiol. 2014;177(3):925–34.
26. Cengiz M, Karatas OF, Koparir E, Yavuzer S, Ali C, Yavuzer H, et al. Differential expression of hypertension-associated microRNAs in the plasma of patients with white coat hypertension. Medicine (Baltimore). 2015;94(13), e693.
27. Williams Z, Ben-Dov IZ, Elias R, Mihailovic A, Brown M, Rosenwaks Z, et al. Comprehensive profiling of circulating microRNA via small RNA sequencing of cDNA libraries reveals biomarker potential and limitations. Proc Natl Acad Sci U S A. 2013;110(11):4255–60.
28. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods. 2013;10(10):1003–5.
29. Marques FZ, Campain AE, Tomaszewski M, Yang YHJ, Zukowska-Sczechowska E, Charchar FJ, et al. Gene expression profiling reveals renin mRNA overexpression in human hypertensive kidneys and a role for microRNAs. Hypertension. 2011;58:1093–8.

30. Tomaszewski M, Charchar FJ, Lynch MD, Padmanabhan S, Wang WY, Miller WH, et al. Fibroblast growth factor 1 gene and hypertension: from the quantitative trait locus to positional analysis. Circulation. 2007;116(17):1915–24.
31. Tomaszewski M, Charchar FJ, Nelson CP, Barnes T, Denniff M, Kaiser M, et al. Pathway analysis shows association between FGFBP1 and hypertension. J Am Soc Nephrol. 2011;22(5):947–55.
32. Eskildsen TV, Jeppesen PL, Schneider M, Nossent AY, Sandberg MB, Hansen PB, et al. Angiotensin II regulates microRNA-132/-212 in hypertensive rats and humans. Int J Mol Sci. 2013;14(6):11190–207.
33. Santovito D, Mandolini C, Marcantonio P, De Nardis V, Bucci M, Paganelli C, et al. Overexpression of microRNA-145 in atherosclerotic plaques from hypertensive patients. Expert Opin Ther Targets. 2013;17(3):217–23.
34. Sethupathy P, Borel C, Gagnebin M, Grant GR, Deutsch S, Elton TS, et al. Human microRNA-155 on chromosome 21 differentially interacts with its polymorphic target in the AGTR1 3' untranslated region: a mechanism for functional single-nucleotide polymorphisms related to phenotypes. Am J Hum Genet. 2007;81(2):405–13.
35. Ceolotto G, Papparella I, Bortoluzzi A, Strapazzon G, Ragazzo F, Bratti P, et al. Interplay between miR-155, AT1R A1166C polymorphism, and AT1R expression in young untreated hypertensives. Am J Hypertens. 2011;24(2):241–6.
36. Elton TS, Sansom SE, Martin MM. Cardiovascular disease, single nucleotide polymorphisms, and the renin angiotensin system: is there a microRNA connection? Int J Hypertens. 2010;2010(2010):281692.
37. Nossent AY, Hansen JL, Doggen C, Quax PH, Sheikh SP, Rosendaal FR. SNPs in microRNA binding sites in 3'-UTRs of RAAS genes influence arterial blood pressure and risk of myocardial infarction. Am J Hypertens. 2011;24(9):999–1006.
38. Maharjan S, Mopidevi B, Kaw MK, Puri N, Kumar A. Human aldosterone synthase gene polymorphism promotes miRNA binding and regulates gene expression. Physiol Genomics. 2014;46(24):860–5.
39. Mopidevi B, Ponnala M, Kumar A. Human angiotensinogen +11525 C/A polymorphism modulates its gene expression through microRNA binding. Physiol Genomics. 2013;45(19): 901–6.
40. Yang Z, Venardos K, Jones E, Morris BJ, Chin-Dusting J, Kaye DM. Identification of a novel polymorphism in the 3'UTR of the L-arginine transporter gene SLC7A1: contribution to hypertension and endothelial dysfunction. Circulation. 2007;115(10):1269–74.
41. Yang Z, Kaye DM. Mechanistic insights into the link between a polymorphism of the 3'UTR of the SLC7A1 gene and hypertension. Hum Mutat. 2009;30(3):328–33.
42. Wang L, Rao F, Zhang K, Mahata M, Rodriguez-Flores JL, Fung MM, et al. Neuropeptide Y(1) Receptor NPY1R discovery of naturally occurring human genetic variants governing gene expression in cella as well as pleiotropic effects on autonomic activity and blood pressure in vivo. J Am Coll Cardiol. 2009;54(10):944–54.
43. O'Connor DT, Zhu G, Rao F, Taupenot L, Fung MM, Das M, et al. Heritability and genome-wide linkage in US and Australian twins identify novel genomic regions controlling chromogranin a: implications for secretion and blood pressure. Circulation. 2008;118(3):247–57.
44. Wei Z, Biswas N, Wang L, Courel M, Zhang K, Soler-Jover A, et al. A common genetic variant in the 3'-UTR of vacuolar H+-ATPase ATP6V0A1 creates a microRNA motif to alter chromogranin A processing and hypertension risk. Circ Cardiovasc Genet. 2011;4(4):381–9.
45. Arora P, Wu C, Khan AM, Bloch DB, Davis-Dusenbery BN, Ghorbani A, et al. Atrial natriuretic peptide is negatively regulated by microRNA-425. J Clin Invest. 2013;123(8):3378–82.
46. Hanin G, Shenhar-Tsarfaty S, Yayon N, Hoe YY, Bennett ER, Sklan EH, et al. Competing targets of microRNA-608 affect anxiety and hypertension. Hum Mol Genet. 2014;23(17):4569–80.

47. Ghanbari M, Franco OH, de Looper H, Hofman A, Erkeland S, Dehghan A. Genetic variations in miRNA binding sites affect miRNA-mediated regulation of several genes associated with cardiometabolic phenotypes. Circ Cardiovasc Genet. 2015;8(3):473–86.
48. Fu X, Guo L, Jiang ZM, Zhao LS, Xu AG. An miR-143 promoter variant associated with essential hypertension. Int J Clin Exp Med. 2014;7(7):1813–7.
49. Ghanbari M, de Vries PS, de Looper H, Peters MJ, Schurmann C, Yaghootkar H, et al. A genetic variant in the seed region of miR-4513 shows pleiotropic effects on lipid and glucose homeostasis, blood pressure, and coronary artery disease. Hum Mutat. 2014;35(12):1524–31.
50. Han F, Konkalmatt P, Chen J, Gildea J, Felder RA, Jose PA, et al. miR-217 Mediates the protective effects of the dopamine d2 receptor on fibrosis in human renal proximal tubule cells. Hypertension. 2015;65(5):1118–25.
51. Ostchega Y, Dillon CF, Hughes JP, Carroll M, Yoon S. Trends in hypertension prevalence, awareness, treatment, and control in older U.S. adults: data from the National Health and Nutrition Examination Survey 1988 to 2004. J Am Geriatr Soc. 2007;55(7):1056–65.
52. Wang H, He T, Wu C, Zhong PS, Cui Y. A powerful statistical method identifies novel loci associated with diastolic blood pressure triggered by nonlinear gene-environment interaction. BMC Proc. 2014;8 Suppl 1:S61.
53. Fernandes T, Magalhaes FC, Roque FR, Phillips MI, Oliveira EM. Exercise training prevents the microvascular rarefaction in hypertension balancing angiogenic and apoptotic factors: role of microRNAs-16, -21, and -126. Hypertension. 2012;59(2):513–20.
54. Sequeira-Lopez ML, Weatherford ET, Borges GR, Monteagudo MC, Pentz ES, Harfe BD, et al. The microRNA-processing enzyme dicer maintains juxtaglomerular cells. J Am Soc Nephrol. 2010;21(3):460–7.
55. Xin M, Small EM, Sutherland LB, Qi X, McAnally J, Plato CF, et al. MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. Genes Dev. 2009;23(18):2166–78.
56. Albinsson S, Skoura A, Yu J, DiLorenzo A, Fernandez-Hernando C, Offermanns S, et al. Smooth muscle miRNAs are critical for post-natal regulation of blood pressure and vascular function. PLoS One. 2011;6(4), e18869.
57. Davern PJ, Nguyen-Huu TP, La Greca L, Abdelkader A, Head GA. Role of the sympathetic nervous system in Schlager genetically hypertensive mice. Hypertension. 2009;54(4):852–9.
58. Jackson KL, Marques FZ, Watson AM, Palma-Rigo K, Nguyen-Huu TP, Morris BJ, et al. A novel interaction between sympathetic overactivity and aberrant regulation of renin by miR-181a in BPH/2J genetically hypertensive mice. Hypertension. 2013;62(4):775–81.
59. Jackson KL, Marques FZ, Nguyen-Huu TP, Stevenson ER, Charchar FJ, Davern PJ, et al. MicroRNA-181a mimic inhibits the renin-angiotensin system and attenuates hypertension in a neurogenic model of hypertension. High Blood Pressure Research Scientific Sessions 2014, San Francisco. 9–12 September 2014.
60. Fan ZD, Zhang L, Shi Z, Gan XB, Gao XY, Zhu GQ. Artificial microRNA interference targeting AT(1a) receptors in paraventricular nucleus attenuates hypertension in rats. Gene Ther. 2012;19(8):810–7.
61. Nossent AY, Eskildsen TV, Andersen LB, Bie P, Bronnum H, Schneider M, et al. The 14q32 microRNA-487b targets the antiapoptotic insulin receptor substrate 1 in hypertension-induced remodeling of the aorta. Ann Surg. 2013;258(5):743–51. discussion 52–3.
62. Cowley Jr AW, Roman RJ, Kaldunski ML, Dumas P, Dickhout JG, Greene AS, et al. Brown Norway chromosome 13 confers protection from high salt to consomic Dahl S rat. Hypertension. 2001;37(2 Part 2):456–61.
63. Liu Y, Taylor NE, Lu L, Usa K, Cowley Jr AW, Ferreri NR, et al. Renal medullary microRNAs in Dahl salt-sensitive rats: miR-29b regulates several collagens and related genes. Hypertension. 2010;55(4):974–82.
64. Castoldi G, Di Gioia CR, Bombardi C, Catalucci D, Corradi B, Gualazzi MG, et al. MiR-133a regulates collagen 1A1: potential role of miR-133a in myocardial fibrosis in angiotensin II-dependent hypertension. J Cell Physiol. 2012;227(2):850–6.

65. Ling S, Nanhwan M, Qian J, Kodakandla M, Castillo AC, Thomas B, et al. Modulation of microRNAs in hypertension-induced arterial remodeling through the β1 and β3-adrenoreceptor pathways. J Mol Cell Cardiol. 2013;65:127–36.
66. Friese RS, Altshuler AE, Zhang K, Miramontes-Gonzalez JP, Hightower CM, Jirout ML, et al. MicroRNA-22 and promoter motif polymorphisms at the Chga locus in genetic hypertension: functional and therapeutic implications for gene expression and the pathogenesis of hypertension. Hum Mol Genet. 2013;22(18):3624–40.
67. Sahu BS, Sonawane PJ, Mahapatra NR. Chromogranin A: a novel susceptibility gene for essential hypertension. Cell Mol Life Sci. 2010;67(6):861–74.
68. Wahlquist C, Jeong D, Rojas-Munoz A, Kho C, Lee A, Mitsuyama S, et al. Inhibition of miR-25 improves cardiac contractility in the failing heart. Nature. 2014;508(7497):531–5.
69. Delles C, McBride MW, Graham D, Padmanabhan S, Dominiczak AF. Genetics of hypertension: from experimental animals to humans. Biochim Biophys Acta. 2010;1802(12):1299–308.
70. Fagard R, Brguljan J, Staessen J, Thijs L, Derom C, Thomis M, et al. Heritability of conventional and ambulatory blood pressures. A study in twins. Hypertension. 1995;26(6 Pt 1):919–24.
71. Snieder H, Harshfield GA, Treiber FA. Heritability of blood pressure and hemodynamics in African- and European-American youth. Hypertension. 2003;41(6):1196–201.
72. Zeegers MP, Rijsdijk F, Sham P, Fagard R, Gielen M, De Leeuw PW, et al. The contribution of risk factors to blood pressure heritability estimates in young adults: the east flanders prospective twin study. Twin Res. 2004;7(3):245–53.
73. Kupper N, Willemsen G, Riese H, Posthuma D, Boomsma DI, de Geus EJ. Heritability of daytime ambulatory blood pressure in an extended twin design. Hypertension. 2005; 45(1):80–5.
74. Wang X, Ding X, Su S, Harshfield G, Treiber F, Snieder H. Genetic influence on blood pressure measured in the office, under laboratory stress and during real life. Hypertens Res. 2011;34(2):239–44.
75. Hottenga JJ, Whitfield JB, de Geus EJ, Boomsma DI, Martin NG. Heritability and stability of resting blood pressure in Australian twins. Twin Res Hum Genet. 2006;9(2):205–9.
76. Lawlor DA, Smith GD. Early life determinants of adult blood pressure. Curr Opin Nephrol Hypertens. 2005;14(3):259–64.
77. Khan NA, Hemmelgarn B, Herman RJ, Rabkin SW, McAlister FA, Bell CM, et al. The 2008 Canadian hypertension education program recommendations for the management of hypertension: part 2-therapy. Can J Cardiol. 2008;24(6):465–75.
78. Hirt MN, Hansen A, Eschenhagen T. Cardiac tissue engineering: state of the art. Circ Res. 2014;114(2):354–67.
79. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, et al. Natural RNA circles function as efficient microRNA sponges. Nature. 2013;495(7441):384–8.
80. Juan L, Wang G, Radovich M, Schneider BP, Clare SE, Wang Y. Potential roles of microRNAs in regulating long intergenic noncoding RNAs. BMC Med Genomics. 2013;6 Suppl 1:S7.
81. Iaconetti C, Gareri C, Polimeni A, Indolfi C. Non-coding RNAs: the “dark matter” of cardiovascular pathophysiology. Int J Mol Sci. 2013;14(10):19987–20018.
82. Gupta SK, Piccoli MT, Thum T. Non-coding RNAs in cardiovascular ageing. Ageing Res Rev. 2014;17C:79–85.

Chapter 12
microRNA and Pulmonary Hypertension

Olivier Boucherat, François Potus, and Sébastien Bonnet

Abstract Pulmonary arterial hypertension (PAH) is a lethal vasculopathy associated with complex etiology that involves remodeling of distal pulmonary arteries leading to elevation of pulmonary vascular resistance. This process results in right ventricular (RV) hypertrophy and ultimately RV failure. In addition, PAH is associated with systemic impairment in the skeletal muscle contributing to exercise intolerance. It has only been a few decades since microRNAs (miRNAs) have been implied in the development and progression of PAH regarding every organ affected by the disease. Indeed, impairment of miRNA's expression has been involved in vascular cell remodeling processes such as adventitial fibroblast (AdvFB) migration; pulmonary arterial smooth muscle cell (PASMC) proliferation and pulmonary arterial endothelial cell (PAEC) dysfunction observed in PAH. At the molecular level miRNAs have been described in the control of ion channels and mitochondrial function as well as the regulation of the BMPR2 signaling pathways contributing to PAH lung impairment. Recently miRNAs have also been specifically implicated in RV dysfunction and systemic angiogenic impairment, observed in PAH. In this chapter, we will summarize the knowledge on miRNA in PAH and highlight their crucial role in the etiology of this disease.

Keywords PAH • microRNA • Lung • Right ventricle • PASMC • PAEC

Introduction

Pulmonary arterial hypertension (PAH) is a debilitating cardiopulmonary disorder of various origins, defined as an increase in pulmonary artery pressure ≥25 mmHg at rest [1]. PAH is classified into five groups based on hemodynamic criteria, etiology, and response to treatments [2]. Although associated with a range of underlying

O. Boucherat
Pulmonary Hypertension Research Group of the University Institute of Cardiology and Pneumology, Québec Research Center, Laval University, Quebec City, QC, Canada

F. Potus • S. Bonnet (✉)
Pulmonary Hypertension Research Group of the Quebec Heart and Lung Institute, Québec Research Center, Laval University, Quebec City, QC, Canada
e-mail: sebastien.bonnet@criucpq.ulaval.ca

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_12

etiologies, all forms of PAH share a common pulmonary arteriopathy characterized by vasoconstriction, remodeling of the precapillary pulmonary vessel wall, and the subsequent obliteration of the vascular lumen. These alterations are responsible for the rise in pulmonary vascular resistance, compensatory right ventricular (RV) hypertrophy, and ultimately death [3]. In addition, PAH is accompanied by significant exercise intolerance contributing to a poor quality of life [4]. Although the pathogenesis of PAH is recognized as a complex and multifactorial process, numerous data have been accumulated demonstrating that endothelial cell dysfunction originating from genetic and environmental factors promotes the release of vasoconstrictors and mitogens that in turn triggers pulmonary vascular cells contraction, proliferation and resistance to apoptosis [5]. Nevertheless, the underlying molecular mechanisms remain elusive. In spite of recent progress in our understanding of the pathophysiological mechanisms involved in this disease and major improvements in symptomatic treatments, no substantial modification of the rapid progression and fatal course of this disease has been achieved. Thus, there is a pressing need to characterize new potential therapeutic targets. The discovery of micro-RNA (miRNA) and their function in regulating gene expression constitutes a major breakthrough in medical science. In regulating all cellular processes in which they have been tested, implication of miRNA in the development and progression of various diseases has gained a lot of interest during the last years, and PAH is no exception. This section summarizes current knowledge regarding the implication of miRNA in signaling pathways involved in PAH pathogenesis (Fig. 12.1).

PAH may occur as a primary rare disease or as a common and severe complication of various cardiac, pulmonary, or systemic conditions. At the histopathological level, PAH is characterized by a thickening of the vessel wall due to abnormal and uncontrolled growth of resident cells. Pulmonary arterial endothelial cell (PAEC) dysfunction is thought to be a key event in the development of PAH in participating to the proliferative and apoptosis-resistant phenotype of pulmonary arterial smooth muscle cells (PASMCs).

Imbalances between circulating and locally produced vasodilatators and vasoconstrictors, mitogenic factors and growth inhibitors, associated with excessive vascular remodeling, are proposed as the underlying processes responsible for the elevated pulmonary vascular resistance seen in PAH [6]. Endothelial injuries, caused by genetic or environmental factors, mechanical stress or immune and inflammatory mechanisms, lead to hypertrophy and hyperplasia of PASMCs. PAECs and PASMCs from PAH patients exhibit dysmorphic and hyperpolarized mitochondria and a glycolytic shift in metabolism, also designated Warburg effect [7]. It has been demonstrated that persistent activation of STAT3 accounts for the activation of several proteins such as HIF1α and NFAT, which in turn triggers the expression of survivin and Pim1, two proto-oncogenes contributing to the metabolic shift and the pro-migratory, pro-proliferative and antiapoptotic phenotype of PAH-PASMC [8–10]. In addition to others factors, inhibition of potassium (K^+) channels and influx of calcium (Ca^{2+}) play a critical role in mediating these effects [11].

Although the pathogenesis of PAH is not completely elucidated and is still explored, compelling evidence indicate that aberrant alterations of miRNA is involved in the development and onset on PAH [12–14].

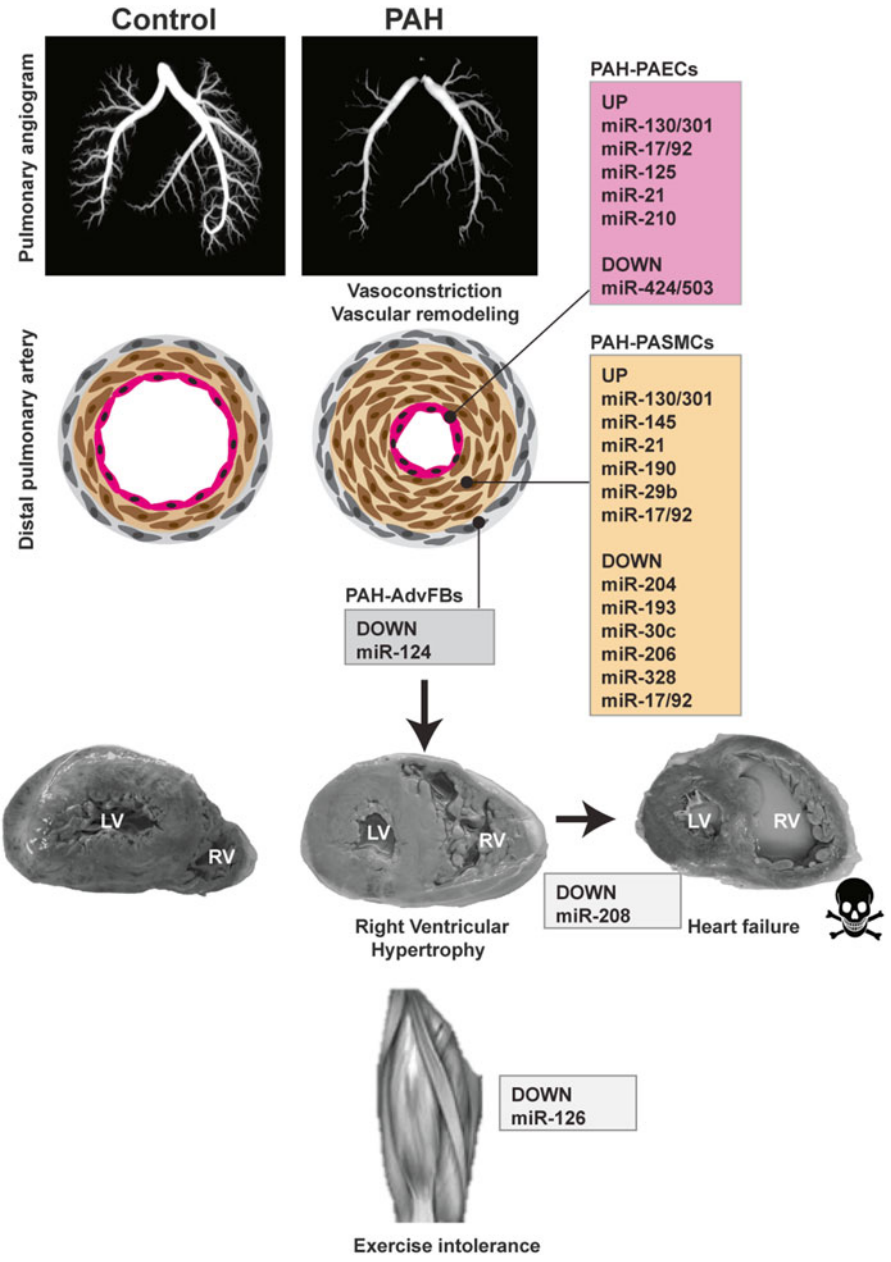

Fig. 12.1 Schematic overview of the miRNAs implicated in PAH etiology. *PAECs* pulmonary arterial endothelial cells, *PASMCs* pulmonary arterial smooth muscle cells, *AdvFBs* adventitial fibroblast, *LV* left ventricle, *RV* right ventricle

microRNA in PAH Lungs

Implication of microRNAs in Endothelial Integrity

BMPR2 Signaling Pathway

The bone morphogenetic signaling received great interest by researchers because of the identification of heterozygous germline mutations in the gene coding for the bone morphogenetic protein (BMP) type-2 receptor (BMPR2), a receptor for the transforming growth factor (TGF)-beta superfamily [15, 16]. The BMPR2 mutation has been found to account for approximately 70 % of familial PAH and for approximately 25 % of idiopathic PAH. The implication of BMPR2 signaling is further strengthened by a case report describing a Smad 8 gene (a downstream mediator of BMPR2) mutation in a patient with sporadic PAH [17]. The penetrance of PAH in patients carrying a BMPR2 mutation is incomplete suggesting that epigenetic or environmental factors influence its effect. This mutation confers a reduction in BMPR2 signaling activity and elicits different effects in cell types. In PASMCs, BMP signals via a Smad-dependent pathway to inhibit proliferation. In PAECs, BMPR2 mutation induces mitochondrial dysfunction, increases the susceptibility of cells to apoptosis that destabilizes the endothelial barrier and triggers a complex cascade of events leading to apoptosis-resistant endothelial cell proliferation and occlusive vascular remodeling (plexiform lesions) [15].

In addition, PAEC dysfunction stimulates the release of vasoconstrictors and growth factors (FGF2, PDGF…) with pro-proliferative effects on PASMCs [18–20]. Mechanistically, reduced BMPR2 signaling disrupts β-catenin/PPARγ interaction leading to the downregulation of apelin (APLN) gene expression. The latter was reported to promote PAEC survival in an autocrine fashion and to exert a paracrine action in inhibiting PASMC growth [21]. In line with these data, Kim and collaborators established an endothelial apelin-FGF link mediated by miR-424 and miR-503 [22]. They confirmed previous findings showing reduced expression of APLN is PAECs of patients with PAH and demonstrated that APLN knockdown in control PAECs promotes the expression of FGF2 and FGFR1 through the downregulation of miR-424/503 [21, 22]. miR-404 and miR-503 negatively regulated PAEC proliferation and inhibited the capacity of PAEC-conditioned medium to induce the proliferation of PASMCs. In direct connection with PAH, miR-424/503 were reduced in PAECs derived from patients with PAH as compared to PAECs from control specimens and FGF2/FGFR1 displayed inverse transcript correlation. Similar results were obtained in experimental PAH models allowing for their use in therapeutic interventions. Sustained elevation of miR-424/503 expression in the experimental PAH models by intranasal lentiviral delivery prevented elevation of right ventricular systolic pressure (an indicator of the severity of PAH) and reversed vascular remodeling. These observations suggest that restoration of miR-424/503 function may provide clinical benefit in PAH [22].

Further miRNAs, such as miR-130/301 family and miR17/92 cluster, have been described to regulate the BMPR2-β-catenin/PPARγ-Apelin signaling pathway and thus to participate in PAH progression. Through a network-based approach to decipher the involvement of miRNAs in PAH pathogenesis, miR-130/301 were identified as critical regulators of vascular homeostasis by inhibiting PPARγ [23, 24]. The miR-130/301 family was upregulated in PAECs under hypoxia and inflammatory stimuli (two known triggers of pulmonary hypertension) as well as in cells transfected with BMPR2 siRNA. The direct repression of PPARγ expression mediated by miR-130/301 upregulation was accompanied by reduced APLN and miR-424/503 expression and increased FGF2. Consistently, miR-130 mimics augmented PAEC proliferation and this effect was reversed by forced expression of PPARγ or miR-424/503. The expression level of miR-130/301 was upregulated in diverse forms of human PAH as well as in multiple models of the disease. Both gain-of-function and loss-of-function approaches were employed to investigate the functional contribution of miR-130/301 in vivo. The authors demonstrated that chronic miR-130/301 instillation in mice is sufficient to induce pulmonary hypertension in a PPARγ-dependent manner. *A contrario*, anti-miR oligonucleotides injections reversed PH in mice [23]. Together, these findings revealed miR-130/301 as a key player in PAH, orchestrating subordinate miRNA and target gene network to control cell proliferation and PAH manifestation in vivo.

A reduced level of BMPR2 signaling in pulmonary arteries (PAECs and PASMCs) is a common denominator of PAH, even when BMPR2 gene mutations were not identified. Given that hypoxia and inflammatory mediators were found to diminish the expression of BMPR2 at the translational level without affecting the levels of corresponding mRNA, a posttranscriptional mechanism of regulation mediated by miRNA was hypothesized [25, 26]. Using bioinformatics tools, several miRNAs encoded by the miR-17/92 polycistronic cluster were retrieved as potential regulators. To confirm the predicted value on a functional basis, BMPR2 protein level was examined after overexpression of the entire cluster. A significant reduction in BMPR2 protein was observed. A more detailed analysis revealed that miR-17-5p and miR-20a were mainly responsible for this modulation. Moreover, the expresses levels of C13orf25 (the primary transcript of miR-17/92) was upregulated by interleukin-6 (IL-6) implicated in the pathogenesis of PAH via the activation of the transcription factor STAT3 [26, 27].

Using an in silico approach aiming at the BMPR2-specific identification of regulatory miRNAs, miR-125a was selected. Consistently with its implication in pulmonary hypertension, levels of miR-125a, highly expressed in endothelial cells, were elevated in lung tissue of hypoxic animals. Functional inhibition of miR-125 in PAECs enhanced proliferation of these cells associated with upregulation of BMPR2 and reduced expression of the tumor suppressors CDKN1A (p21) and CDKN2A (p16) [28]. Accordingly, transfection with miR-125a inhibitor enhanced human umbilical vascular endothelial cells (HUVECs) proliferation and resistance to apoptosis, and promoted tube formation in Matrigel, suggesting that reduction of miR-125a has a pro-angiogenic effect [29].

A central regulatory role of miR-21, abundantly expressed in the endothelium, was delineated by several studies. Hypoxia and BMPR2 signaling independently upregulated miR-21 in human PAECs and paradoxically, forced expression of miR-21 decreased BMPR2 expression highlighting a reciprocal feedback loop [29, 30]. Interestingly, miR-21 processing was completely blocked by knockdown of the BMPR2 downstream mediator smad8 [31]. Furthermore, miR-21 was shown to directly repress Rho kinase activity favoring vasodilatation. As suggested by a network bioinformatics approach, miR-21 was increased in remodeled pulmonary vessels of animal models of PAH and human PAH [30, 32]. Nevertheless, compared to control littermates, miR-21$^{-/-}$ mice displayed exaggerated manifestations of pulmonary hypertension when exposed to a combination of Sugen5416 and chronic hypoxia [30]. The detrimental effects of miR-21 loss of function under pathological conditions appears to result from the functional induction of programmed cell death 4 (PDCD4, a tumor suppressor)/caspase-3 axis. In serum-deficient PAECs, miR-21 mimic transfection repressed induction of its target PDCD4, to consequently inhibit caspase-3 activation. Likewise, in vivo miR-21 silencing by chronic delivery of anti-miR-21 or by genetic inactivation (miR-21$^{-/-}$ mice) was accompanied by elevated PDCD4 levels coupled with the enhanced detection of cleaved caspase-3. Consistently with prior findings, miR-21 loss of function caused the progressive onset of PAH in vivo, a phenotype abolished in mice overexpressing miR-21 [33]. From these observations, induced miR-21 expression in PAECs could be interpreted as a rescue mechanism to counteract progression of PAH.

Collectively, these data provide a view of the combinatorial effort of miRNAs to control the BMPR2 signaling pathway at different levels to impact endothelial cell behavior. Reciprocally, BMPR2 signaling impacts miRNA processing.

miRNA-210 and Mitochondrial Dysfunction

Besides miR-424/503 and miR-130/301, a)direct mechanistic)evidence for the role of miR-210 in PAH was highlighted. miR-210 was found robustly induced by hypoxia in human PAECs. The authors reported that miR-210 directly repressed the expression of iron-sulfur cluster assembly proteins (ISCU1/2), controlling mitochondrial metabolic functions [34]. Based on current knowledge pinpointing mitochondrial dysfunction and iron homeostasis as key factors in PAH development [35–37], White and collaborators demonstrated that alterations of the miR-210-ISCU1/2 axis cause iron-sulfur deficiencies in vivo and promote pulmonary hypertension. Importantly, the authors showed that pulmonary vascular overexpression of miR-210, achieved by intrapharyngial delivery of miR-210 mimic, represses ISCU1/2 levels and induces vascular remodeling reminiscent of PAH. These abnormalities were phenocopied after ISCU1/2 knockdown underscoring the importance of ISCU1/2 and iron-sulfur biogenesis in PAH. Finally, they undertook a translational study using preclinical models of PAH to address the potential therapeutic benefit of miR-210 inhibition. The authors reported that the genetic inactivation of miR-210 protects mice against the development of PAH and corroborated these

results in a pharmacological approach using miR-210 inhibitors [38]. Together, these eloquent results provide a solid foundation for the initiation of human clinical trials in human PAH.

Implication of microRNAs in the Pro-proliferative and Apoptosis-Resistant Phenotype of PAH PASMCs

As stated above, one of the most prominent features of PAH is the medial hypertrophy and adventitial thickening of pulmonary arteries due to the pro-proliferative and antiapoptotic phenotype of PASMCs. Although these defects have been the target of considerable research and clinical attention, the underlying mechanisms remain incompletely understood. It is nonetheless established that several signaling pathways are responsible for the cancer-like phenotype of PAH PASMCs. Alterations of numerous miRNA in targeting several members of a given pathway or multiple targets in converging pathways have been reported to impact PASMC behavior.

There is substantial evidence that place the signal transducer and activator of transcription 3 (STAT3) at the center of a hub for integrating different signals and regulating energy metabolism, growth, and survival in PASMCs [39–41]. In PAH-PASMCs, STAT3 activation in response to a wide array of stimuli such as IL-6, tumor necrosis factor alpha (TNFα), endothelin-1 (ET1), and PDGF is mediated through the Janus family kinase (JAK) or Src. Persistent activation of STAT3 is a hallmark of PAH and participate to the activation of NFAT, HIF1α, Pim1, KLF5/survivin, all involved in aberrant PASMC proliferation and resistance to apoptosis [40, 42, 43]. Comparison of miRNA expression profiles between PASMCs isolated from distal pulmonary arteries of PAH patients and healthy donors revealed a marked depression of miR-204 expression in PAH. Mainly restricted to PASMCs in the lung, miR-204 was found systematically reduced in human PAH and rodent models of the disease. The study demonstrated that STAT3 activation suppresses miR-204 expression, and miR-204 directly targets SHP2, a positive regulator of SRC family kinase activity. Thus, through the downregulation of miR-204 and the upregulation of SHP2, STAT3 enhances a positive feedback loop sustaining its own activation [41]. Further analysis demonstrated that miR-204 restoration in PAH-PASMCs was associated with decreased $[Ca^{2+}]i$ and depolarization of the mitochondrial membrane potential reversing the pro-proliferative and antiapoptotic phenotype of PAH-PASMCs. A major strength of the study was the finding that nebulization of synthetic miR-204 in rats with established monocrotaline (MCT)-induced PAH can reverse symptoms of the disease, as illustrated by reduced medial wall thickness and improvement in mean pulmonary artery pressure. In an opposite manner, miR-204 antagomiR nebulization in control rats induced PAH development [41]. It was also observed that sustained DNA damage and miR-223 defect observed in PAH-PASMCs accounts for a poly [ADP-ribose] polymerase 1 (PARP-1)-dependent downregulation of miR-204 [44, 45]. Moreover, miR-130/301 involved in the repression of

miR-424/503 in PAECs also suppresses miR-204 in PASMCs indicating that miR-204 is subject to multiple regulatory systems [21]. These in-depth studies incriminate suppression of miR-204 expression as a cardinal contributor of PAH.

Reduced pulmonary expression of miR-30c and miR-206 is also a feature of mice exposed to hypoxia. After in vitro inhibition of miR-30c, control PASMCs adopted a PAH-PASMC like phenotype characterized by enhanced cell proliferation and reduced apoptosis. Mechanically, the authors demonstrated that these effects are mediated through the inhibition of platelet-derived growth factor receptor beta (PDGFRβ) expression by directly binding to the 3′ untranslated region of the transcript [46]. Similar conclusions were reached for miR-206, except that the latter was shown to reduce NOTCH3 expression [47]. Although the contribution of miR-30c and miR-206 has not been explored in experimental PAH models, the demonstrated implication of PDGF and NOTCH signaling in PAH supports the notion that miR-30c and miR-206 may represent two new therapeutic targets in hypoxia-induced PAH [48–50].

miR-145 expression was specifically localized by in situ hybridization within the smooth muscle layer of vessels and bronchi of control mice. Its robust upregulation under hypoxia exposure suggested a role of the latter in PAH. Its contribution in PAH was examined using genetically modified mice. Compared to wild-type mice, miR-145$^{-/-}$ mice did not display systolic right ventricular pressure and right ventricular hypertrophy in response to hypoxia. Pulmonary vascular remodeling was also reduced in these mice indicating protection against the development of PAH. These findings were replicated by pharmacological manipulation of miR-145 using a specific antagomiR. In human pulmonary arteries, miR-145 was confined to PASMCs and significantly elevated in lungs of patients with primary PAH, confirming animal model findings. Whereas anti-miR-driven downregulation or miR-145 mimic transfection failed to influence BMPR2 expression in PASMCs, siRNA knockdown of BMPR2 induced a marked upregulation of miR-145, indicating that miR-145 acts downstream of BMPR2 signaling. Consistently, mice harboring a heterozygous BMPR2 tail domain mutation (BMPR2$^{R899X/+}$) and spontaneously developing PAH presented high expression levels of miR-145 [51].

As observed in PAECs, miR-21 expression is also upregulated in PASMCs after hypoxia exposure. miR-21 is essential for hypoxia-induced cell proliferation, resistance to apoptosis, and migration. Expression of PDCD4 and sprouty homolog 2 (SPRY2, a suppressor of Ras/MAPK signaling), two target genes of miR-21, were decreased in hypoxia and in PASMCs overexpressing miR-21 in normoxia and increased in hypoxic cells in which miR-21 was knocked down [32, 52]. Taken together, these data underscore a significant role of miR-21 in hypoxia-induced PASMCs proliferation and migration by regulating multiple gene targets. Since PDCD4 is also a target of miR-21 is PAECs, these data indicate that miR-21 exerts similar effects independently of the cell type (PAECs or PASMCs). However, contrary to PAECs, the upregulation of miR-21 in PASMCs appears harmful [33]. The opposite roles of miR-21 in these different cell types may explain conflicting results obtained in PAH animal models after miR-21 knockdown [30, 32, 33].

Based on the fact that (1) elevated expression of miR-17 and miR-20a belonging to the miR-17/92 cluster was evoked to be responsible for the abnormal phenotype of PAH-PASMCs and (2) enforced expression of miR-17/92 exhibits strong tumorigenic activity in multiple models, Chen and collaborators examined the PASMC-specific contribution of the cluster to the pathogenesis of PAH [53, 54]. To do so, a mouse model of conditional inactivation of miR-17/92 in smooth muscle cells was generated. In vivo targeted deletion of miR-17/92 attenuated the hypoxia-induced increases in right ventricular pressure, right ventricular hypertrophy, and pulmonary arterial thickness. This beneficial effect was explained by in vitro findings showing that miR-17/92 lentiviral infection promotes proliferation and smooth muscle cell markers (αSMA, Calponin, SM22α) expression in normal PASMCs. This study identified PDZ and LIM domain protein 5 (PDLIM5) as a negative regulator of SMC-differentiated phenotype via a TGFβ3/Smad3 signaling and a direct target of miR-17/92 in human PASMC. The authors reported a biphasic regulation of miR-17/92 expression characterized by an early transient induction and a late phase reduction potentially reflecting a first causal and a subsequent adaptive response to inhibit further progression of pulmonary hypertension [55]. This temporal divergence during PAH progression needs to be further clarified.

microRNAs and Ion Channels in PAH

Implication of K^+ channels) (especially voltage-gated and two pore K^+ channels) in the)etiology pulmonary hypertension has been)described around 20 years ago [56]. Inhibition of K^+ channels secondary to hypoxia or key pathogenic factors (such as endothelin-1 and serotonin) causes cell depolarization and calcium entry. Increased levels of intracellular K^+ and Ca^{2+} triggers pulmonary vasoconstriction and stimulate PASMC proliferation as well as resistance to apoptosis. Initially selected for their upregulation under hypoxia, miR-190 and miR-29b are mainly expressed in PASMCs. Voltage-gated potassium channels Kv7.5 and Kv1.5 were found to be regulated by miR-190 and miR-29b, respectively [57, 58]. miR-190 transfection remarkably enhanced the vasoconstriction responses to phenylephrine and KCl of endothelium-denuded pulmonary artery rings and produced an increase in cytosolic free Ca^{2+} concentration in PASMCs [57]. Contrary to miR-190 and miR-29b, miR-328 showed opposite changes in expression with a significant diminution in rat subjected to hypoxia and in pulmonary arteries (PA) from PAH patients [59]. Under both normoxic and hypoxic conditions, transgenic mice overexpressing miR-328 under the control of smooth muscle-myosin heavy chain (SM-MHC) regulatory sequences exhibited reduced right ventricular systolic pressure and wall thickness compared to wild-type mice. In rat PASMCs, overexpression of miR-328 induced cell apoptosis and western blot analysis coupled with luciferase reporter assays established that miR-328 targets insulin growth factor 1 receptor (IGF1R) and the L-type calcium channel α1C (CaV1.2), a major Ca^{2+} entry pathway in

smooth muscle cells. As CaV1.2 expression is reduced in PA from hypoxic rats, the study provided direct evidences that chronic hypoxia induced PA constriction and remodeling via a miR-328/CaV1.2 axis [59].

Protective Role of miR-193 on PAH-Induced Pulmonary and Cardiac Abnormalities

Biological metabolites of arachidonic and linoleic acids, such as hydroxyeicosatetraenoic (HETE) and hydroxyoctadecadienoic (HODE) acids, have been found to stimulate growth and resistance to apoptosis in many cell types including pulmonary vascular smooth muscle cells [60, 61]. Apolipoprotein A-I (apoA-I) mimetic peptides (4F) that bind proinflammatory lipids and decrease their levels have been developed and displayed therapeutic potential for reversing atherosclerosis in animal models and humans [62]. Based on biological properties of 4F, Sharma and collaborators examined the potential benefit of 4F treatment in PAH models. In their study, 4F was shown to rescue advanced PAH by improving cardiac and pulmonary structure and function. The corrective effects of 4F were accompanied by a restoration of miR-193 levels, diminished in lung and plasma of PAH patients and models. As stated for miR-328 (see above), miR-193 directly targets IGF1R, and its overexpression in PAH-PASMCs tempered proliferation. Intratracheal administration of miR-193 mimics was shown to reverse symptoms of PAH on both MCT and hypoxia models highlighting the direct contribution of miR-193 in the beneficial effects mediated by 4F. Finally, the authors revealed that HETE and HODE acids induce the expression of the retinoid X receptor alpha which silence miR-193 expression by binding to its promoter [63].

miRNA-124 Governs the Proliferative, Migratory, and Inflammatory Phenotype of PAH-Fibroblasts

In addition to endothelial and smooth muscle cells, adventitial fibroblast (AdvFBs) is another major cell type deregulated in PAH. Insights have emphasized the role of AdvFBs in controlling arterial remodeling through the liberation of cytokines, chemokines, and mitogenic factors but relatively few studies have investigated the role of miRNAs in the acquired and stable changes of this phenotype [64]. In a candidate approach based on literature, the contribution of miR-124 in the activated phenotype of pulmonary AdvFBs was explored. As expected, a robust decrease expression of miR-124 was noted in highly proliferative and promigratory PAH-AdvFBs compared to control fibroblasts. These parameters were attenuated following miR-124 expression.

The repressive action of miR-124 on polypyrimidine tract-binding protein 1 (PTBP1) and monocyte chemoattractant protein 1 (MCP-1/CCL2) was demonstrated

as the causal mechanism responsible for the heightened proliferative, migratory, and inflammatory properties of PAH-AdvFBs. This study provided other important information. The authors reported no decrease in miR-124 expression in different rodent models of PH, underscoring inter-species miR-124 expression divergence. Moreover, they pinpointed histone deacetylases as repressors of miR-124 expression [65].

microRNAs and PAH Right Ventricle

Right ventricle failure (RVF) is the primary cause of death in pulmonary hypertension patients [66]. For many years, clinicians have unsuccessfully extrapolated knowledge from the left ventricular (LV) failure to the RV. This lack of success is likely due to the differences in the embryologic origin, metabolic profile or response to pressure overload existing between the RV and the LV [67]. As a result, RV failure has emerged as an important research priority in the cardiopulmonary research field. It is only recently that the role of miRNAs in RV failure has been explored, and if many miRNAs [68] have been showed to be mis-regulated in RV failure, only one has been extensively studied by Paulin et al. in 2015 [69]. Indeed, in their study they showed for the first time a critical role of miR-208/Mef2 axis in RVF. In contrast to the left ventricle failure, the level of the myocardium specific miRNA miR-208 continuously decreases as RVH progresses, eventually allowing repression of MEF2 transcription factor and thus the transition from a compensated RV hypertrophy to a decompensated RVH. In addition, they provide evidences that inflammation (which is a major players in PAH) could serve as second trigger for entrance in decompensated RV hypertrophy in synergy with the progressive lost of miR-208.

microRNAs and Systemic Angiogenic Impairment in PAH

Interestingly, preliminary reports have also suggested a role for miRNAs regulating angiogenesis known as angiomiR in PAH, including miR-126 [70]. Indeed, lost of angiogenesis is an important feature implicated in both cardiac and skeletal muscle dysfunction as seen in RVF and exercise intolerance in PAH [71]. Potus and collaborators have reported in muscle biopsies obtained from patients with PAH the existence of an inverse correlation between reduced muscle capillarity and exercise capacity. The authors provided evidences that the downregulation of angiogenesis in skeletal muscle was due to downregulation of the vascular endothelial growth factor pathway, a master regulator of systemic angiogenesis following the lost of miR-126, a miRNA that is involved in both embryonic and adult angiogenesis. Using a combination of in vitro and in vivo techniques, the authors showed that increasing doses of miR-126 were able to restore proper vascularization in vitro and that direct delivery of miR-126 to muscle can increase both vascularization and

performance, ultimately leading to a substantial improvement in exercise capacity. Recently, the same group has identified miR-126 downregulation as a central actor of angiogenic defect which is crucial for RVF [72].

Conclusion

In the field of PAH, considerable efforts have been committed to identify miRNAs and decipher their functions. As PAH is a multifactorial disease involving dysfunction of several cell types, it is not surprising that a plethora of miRNAs are involved in the disease. Most of the studies analyzed the repercussions of selected miRNA alterations but not the mechanisms responsible for regulation of the expression of miRNAs themselves. Most of them have been described as being influenced by hypoxia. Therefore, the identification of HIF regulated miRNAs will undoubtedly provide important insights. Although the instrumental roles of these miRNAs in PAH pathogenesis are beginning to be unveiled, a major importance of the latter is already proved in the disease, making miRNA a promising therapeutic target in human. Body fluids-expressed miRNAs are reliable PAH diagnostic biomarkers [73]. However, it will be interesting to evaluate whether circulating miRNAs can be considered in animal models as treatment-response predictors. Taken together, published data indicate that miRNAs have a great potential as therapeutics in PAH.

References

1. Badesch DB, Champion HC, Sanchez MAG, Hoeper MM, Loyd JE, Manes A, et al. Diagnosis and assessment of pulmonary arterial hypertension. J Am Coll Cardiol. 2009;54(1 Suppl):S55–66.
2. Simonneau G, Robbins IM, Beghetti M, Channick RN, Delcroix M, Denton CP, et al. Updated clinical classification of pulmonary hypertension. J Am Coll Cardiol. 2009;54(1 Suppl):S43–54.
3. Vonk-Noordegraaf A, Haddad F, Chin KM, Forfia PR, Kawut SM, Lumens J, et al. Right heart adaptation to pulmonary arterial hypertension: physiology and pathobiology. J Am Coll Cardiol. 2013;62(25 Suppl):D22–33.
4. Fowler RM, Gain KR, Gabbay E. Exercise intolerance in pulmonary arterial hypertension. Pulm Med. 2012;2012:359204.
5. Hoeper MM, Barberà JA, Channick RN, Hassoun PM, Lang IM, Manes A, et al. Diagnosis, assessment, and treatment of non-pulmonary arterial hypertension pulmonary hypertension. J Am Coll Cardiol. 2009;54(1 Suppl):S85–96.
6. Tuder RM, Archer SL, Dorfmüller P, Erzurum SC, Guignabert C, Michelakis E, et al. Relevant issues in the pathology and pathobiology of pulmonary hypertension. J Am Coll Cardiol. 2013;62(25 Suppl):D4–12.
7. Cottrill KA, Chan SY. Metabolic dysfunction in pulmonary hypertension: the expanding relevance of the Warburg effect. Eur J Clin Invest. 2013;43(8):855–65.
8. Bonnet S, Michelakis ED, Porter CJ, Andrade-Navarro MA, Thébaud B, Bonnet S, et al. An abnormal mitochondrial-hypoxia inducible factor-1alpha-Kv channel pathway disrupts oxy-

gen sensing and triggers pulmonary arterial hypertension in fawn hooded rats: similarities to human pulmonary arterial hypertension. Circulation. 2006;113(22):2630–41.
9. McMurtry MS, Archer SL, Altieri DC, Bonnet S, Haromy A, Harry G, et al. Gene therapy targeting survivin selectively induces pulmonary vascular apoptosis and reverses pulmonary arterial hypertension. J Clin Invest. 2005;115(6):1479–91.
10. Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R, et al. A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. Cancer Cell. 2007;11(1):37–51.
11. Kuhr FK, Smith KA, Song MY, Levitan I, Yuan JX-J. New mechanisms of pulmonary arterial hypertension: role of Ca^{2+} signaling. Am J Physiol Heart Circ Physiol. 2012;302(8): H1546–62.
12. Meloche J, Paulin R, Provencher S, Bonnet S. Therapeutic potential of microRNA modulation in pulmonary arterial hypertension. Curr Vasc Pharmacol. 2015;13(3):331–40.
13. Zhou G, Chen T, Raj JU. MicroRNAs in pulmonary arterial hypertension. Am J Respir Cell Mol Biol. 2015;52(2):139–51.
14. Caruso P, MacLean MR, Khanin R, McClure J, Soon E, Southgate M, et al. Dynamic changes in lung microRNA profiles during the development of pulmonary hypertension due to chronic hypoxia and monocrotaline. Arterioscler Thromb Vasc Biol. 2010;30(4):716–23.
15. Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, et al. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet. 2000;67(3):737–44.
16. Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA, Loyd JE, et al. Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. Nat Genet. 2000;26(1):81–4.
17. Shintani M, Yagi H, Nakayama T, Saji T, Matsuoka R. A new nonsense mutation of SMAD8 associated with pulmonary arterial hypertension. J Med Genet. 2009;46(5):331–7.
18. Izikki M, Guignabert C, Fadel E, Humbert M, Tu L, Zadigue P, et al. Endothelial-derived FGF2 contributes to the progression of pulmonary hypertension in humans and rodents. J Clin Invest. 2009;119(3):512–23.
19. Teichert-Kuliszewska K, Kutryk MJB, Kuliszewski MA, Karoubi G, Courtman DW, Zucco L, et al. Bone morphogenetic protein receptor-2 signaling promotes pulmonary arterial endothelial cell survival: implications for loss-of-function mutations in the pathogenesis of pulmonary hypertension. Circ Res. 2006;98(2):209–17.
20. Yang X, Long L, Reynolds PN, Morrell NW. Expression of mutant BMPR-II in pulmonary endothelial cells promotes apoptosis and a release of factors that stimulate proliferation of pulmonary arterial smooth muscle cells. Pulm Circ. 2011;1(1):103–10.
21. Alastalo T-P, Li M, Perez Vde J, Pham D, Sawada H, Wang JK, et al. Disruption of PPARγ/β--catenin-mediated regulation of apelin impairs BMP-induced mouse and human pulmonary arterial EC survival. J Clin Invest. 2011;121(9):3735–46.
22. Kim J, Kang Y, Kojima Y, Lighthouse JK, Hu X, Aldred MA, et al. An endothelial apelin-FGF link mediated by miR-424 and miR-503 is disrupted in pulmonary arterial hypertension. Nat Med. 2013;19(1):74–82.
23. Bertero T, Lu Y, Annis S, Hale A, Bhat B, Saggar R, et al. Systems-level regulation of microRNA networks by miR-130/301 promotes pulmonary hypertension. J Clin Invest. 2014;124(8):3514–28.
24. Ameshima S, Golpon H, Cool CD, Chan D, Vandivier RW, Gardai SJ, et al. Peroxisome proliferator-activated receptor gamma (PPARgamma) expression is decreased in pulmonary hypertension and affects endothelial cell growth. Circ Res. 2003;92(10):1162–9.
25. Takahashi H, Goto N, Kojima Y, Tsuda Y, Morio Y, Muramatsu M, et al. Downregulation of type II bone morphogenetic protein receptor in hypoxic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol. 2006;290(3):L450–8.
26. Brock M, Trenkmann M, Gay RE, Michel BA, Gay S, Fischler M, et al. Interleukin-6 modulates the expression of the bone morphogenic protein receptor type II through a novel STAT3-microRNA cluster 17/92 pathway. Circ Res. 2009;104(10):1184–91.

27. Kherbeck N, Tamby MC, Bussone G, Dib H, Perros F, Humbert M, et al. The role of inflammation and autoimmunity in the pathophysiology of pulmonary arterial hypertension. Clin Rev Allergy Immunol. 2013;44(1):31–8.
28. Huber LC, Ulrich S, Leuenberger C, Gassmann M, Vogel J, von Blotzheim LG, et al. MicroRNA-125a in pulmonary hypertension: regulator of a proliferative phenotype of endothelial cells. Exp Biol Med (Maywood). 2015;pii, 1535370215579018.
29. Svensson D, Gidlöf O, Turczyńska KM, Erlinge D, Albinsson S, Nilsson B-O. Inhibition of microRNA-125a promotes human endothelial cell proliferation and viability through an anti-apoptotic mechanism. J Vasc Res. 2014;51(3):239–45.
30. Parikh VN, Jin RC, Rabello S, Gulbahce N, White K, Hale A, et al. MicroRNA-21 integrates pathogenic signaling to control pulmonary hypertension: results of a network bioinformatics approach. Circulation. 2012;125(12):1520–32.
31. Drake KM, Zygmunt D, Mavrakis L, Harbor P, Wang L, Comhair SA, et al. Altered microRNA processing in heritable pulmonary arterial hypertension: an important role for Smad-8. Am J Respir Crit Care Med. 2011;184(12):1400–8.
32. Yang S, Banerjee S, de Freitas A, Cui H, Xie N, Abraham E, et al. miR-21 regulates chronic hypoxia-induced pulmonary vascular remodeling. Am J Physiol Lung Cell Mol Physiol. 2012;302(6):L521–9.
33. White K, Dempsie Y, Caruso P, Wallace E, McDonald RA, Stevens H, et al. Endothelial apoptosis in pulmonary hypertension is controlled by a microRNA/programmed cell death 4/caspase-3 axis. Hypertension. 2014;64(1):185–94.
34. Chan SY, Zhang Y-Y, Hemann C, Mahoney CE, Zweier JL, Loscalzo J. MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2. Cell Metab. 2009;10(4):273–84.
35. Xu W, Koeck T, Lara AR, Neumann D, DiFilippo FP, Koo M, et al. Alterations of cellular bioenergetics in pulmonary artery endothelial cells. Proc Natl Acad Sci U S A. 2007;104(4):1342–7.
36. Ryan JJ, Archer SL. Emerging concepts in the molecular basis of pulmonary arterial hypertension: part I: metabolic plasticity and mitochondrial dynamics in the pulmonary circulation and right ventricle in pulmonary arterial hypertension. Circulation. 2015;131(19):1691–702.
37. Cotroneo E, Ashek A, Wang L, Wharton J, Dubois O, Bozorgi S, et al. Iron homeostasis and pulmonary hypertension: iron deficiency leads to pulmonary vascular remodeling in the rat. Circ Res. 2015;116(10):1680–90.
38. White K, Lu Y, Annis S, Hale AE, Chau BN, Dahlman JE, et al. Genetic and hypoxic alterations of the microRNA-210-ISCU1/2 axis promote iron-sulfur deficiency and pulmonary hypertension. EMBO Mol Med. 2015;7(6):695–713.
39. Paulin R, Meloche J, Bonnet S. STAT3 signaling in pulmonary arterial hypertension. JAKSTAT. 2012;1(4):223–33.
40. Paulin R, Courboulin A, Meloche J, Mainguy V, Dumas de la Roque E, Saksouk N, et al. Signal transducers and activators of transcription-3/pim1 axis plays a critical role in the pathogenesis of human pulmonary arterial hypertension. Circulation. 2011;123(11):1205–15.
41. Courboulin A, Paulin R, Giguère NJ, Saksouk N, Perreault T, Meloche J, et al. Role for miR-204 in human pulmonary arterial hypertension. J Exp Med. 2011;208(3):535–48.
42. Courboulin A, Tremblay VL, Barrier M, Meloche J, Jacob MH, Chapolard M, et al. Krüppel-like factor 5 contributes to pulmonary artery smooth muscle proliferation and resistance to apoptosis in human pulmonary arterial hypertension. Respir Res. 2011;12:128.
43. Bonnet S, Rochefort G, Sutendra G, Archer SL, Haromy A, Webster L, et al. The nuclear factor of activated T cells in pulmonary arterial hypertension can be therapeutically targeted. Proc Natl Acad Sci U S A. 2007;104(27):11418–23.
44. Meloche J, Pflieger A, Vaillancourt M, Paulin R, Potus F, Zervopoulos S, et al. Role for DNA damage signaling in pulmonary arterial hypertension. Circulation. 2014;129(7):786–97.
45. Meloche J, Le Guen M, Potus F, et al. miR-223 reverses experimental pulmonary arterial hypertension. Am J Physiol Cell Physiol 2015;309:C363–72. doi: 10.1152/ajpcell.00149.2015.

46. Xing Y, Zheng X, Li G, Liao L, Cao W, Xing H, et al. MicroRNA-30c contributes to the development of hypoxia pulmonary hypertension by inhibiting platelet-derived growth factor receptor β expression. Int J Biochem Cell Biol. 2015;64:155–66.
47. Jalali S, Ramanathan GK, Parthasarathy PT, Aljubran S, Galam L, Yunus A, et al. Mir-206 regulates pulmonary artery smooth muscle cell proliferation and differentiation. PLoS One. 2012;7(10):e46808.
48. Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, et al. Reversal of experimental pulmonary hypertension by PDGF inhibition. J Clin Invest. 2005;115(10):2811–21.
49. Perros F, Montani D, Dorfmüller P, Durand-Gasselin I, Tcherakian C, Le Pavec J, et al. Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension. Am J Respir Crit Care Med. 2008;178(1):81–8.
50. Li X, Zhang X, Leathers R, Makino A, Huang C, Parsa P, et al. Notch3 signaling promotes the development of pulmonary arterial hypertension. Nat Med. 2009;15(11):1289–97.
51. Caruso P, Dempsie Y, Stevens HC, McDonald RA, Long L, Lu R, et al. A role for miR-145 in pulmonary arterial hypertension: evidence from mouse models and patient samples. Circ Res. 2012;111(3):290–300.
52. Sarkar J, Gou D, Turaka P, Viktorova E, Ramchandran R, Raj JU. MicroRNA-21 plays a role in hypoxia-mediated pulmonary artery smooth muscle cell proliferation and migration. Am J Physiol Lung Cell Mol Physiol. 2010;299(6):L861–71.
53. Pullamsetti SS, Doebele C, Fischer A, Savai R, Kojonazarov B, Dahal BK, et al. Inhibition of microRNA-17 improves lung and heart function in experimental pulmonary hypertension. Am J Respir Crit Care Med. 2012;185(4):409–19.
54. Brock M, Samillan VJ, Trenkmann M, Schwarzwald C, Ulrich S, Gay RE, et al. AntagomiR directed against miR-20a restores functional BMPR2 signalling and prevents vascular remodeling in hypoxia-induced pulmonary hypertension. Eur Heart J. 2014;35(45):3203–11.
55. Chen T, Zhou G, Zhou Q, Tang H, Ibe JCF, Cheng H, et al. Loss of microRNA-17∼92 in smooth muscle cells attenuates experimental pulmonary hypertension via induction of PDZ and LIM domain 5. Am J Respir Crit Care Med. 2015;191(6):678–92.
56. Mandegar M, Yuan JXJ. Role of K+ channels in pulmonary hypertension. Vascul Pharmacol. 2002;38(1):25–33.
57. Li S-S, Ran Y-J, Zhang D-D, Li S-Z, Zhu D. MicroRNA-190 regulates hypoxic pulmonary vasoconstriction by targeting a voltage-gated K^+ channel in arterial smooth muscle cells. J Cell Biochem. 2014;115(6):1196–205.
58. Pohl NM, Yamamura A, Yamamura H, Makino A, Yuan JX-J. MicroRNA 29b is upregulated in pulmonary artery smooth muscle cells from patients with idiopathic pulmonary arterial hypertension and inhibits K+ channel expression and function. FASEB J. 2012;26:884.
59. Guo L, Qiu Z, Wei L, Yu X, Gao X, Jiang S, et al. The microRNA-328 regulates hypoxic pulmonary hypertension by targeting at insulin growth factor 1 receptor and L-type calcium channel-α1C. Hypertension. 2012;59(5):1006–13.
60. Zhang L, Ma J, Li Y, Guo L, Ran Y, Liu S, et al. 15-Hydroxyeicosatetraenoic acid (15-HETE) protects pulmonary artery smooth muscle cells against apoptosis via HSP90. Life Sci. 2010;87(7–8):223–31.
61. Ma C, Li Y, Ma J, Liu Y, Li Q, Niu S, et al. Key role of 15-lipoxygenase/15-hydroxyeicosatetraenoic acid in pulmonary vascular remodeling and vascular angiogenesis associated with hypoxic pulmonary hypertension. Hypertension. 2011;58(4):679–88.
62. Sherman CB, Peterson SJ, Frishman WH. Apolipoprotein A-I mimetic peptides: a potential new therapy for the prevention of atherosclerosis. Cardiol Rev. 2010;18(3):141–7.
63. Sharma S, Umar S, Potus F, Iorga A, Wong G, Meriwether D, et al. ApoA-I mimetic peptide 4F rescues pulmonary hypertension by inducing microRNA-193-3p. Circulation. 2014;130(9):776–85.
64. Stenmark KR, Frid MG, Yeager M, Li M, Riddle S, McKinsey T, et al. Targeting the adventitial microenvironment in pulmonary hypertension: a potential approach to therapy that considers epigenetic change. Pulm Circ. 2012;2(1):3–14.

65. Wang D, Zhang H, Li M, Frid MG, Flockton AR, McKeon BA, et al. MicroRNA-124 controls the proliferative, migratory, and inflammatory phenotype of pulmonary vascular fibroblasts. Circ Res. 2014;114(1):67–78.
66. Bogaard HJ, Abe K, Vonk Noordegraaf A, Voelkel NF. The right ventricle under pressure: cellular and molecular mechanisms of right-heart failure in pulmonary hypertension. Chest. 2009;135(3):794–804.
67. Haddad F, Doyle R, Murphy DJ, Hunt SA. Right ventricular function in cardiovascular disease, part II: pathophysiology, clinical importance, and management of right ventricular failure. Circulation. 2008;117(13):1717–31.
68. Thum T, Batkai S. MicroRNAs in right ventricular (dys)function (2013 Grover conference series). Pulm Circ. 2014;4(2):185–90.
69. Paulin R, Sutendra G, Gurtu V, Dromparis P, Haromy AS, Provencher S, et al. A miR-208-Mef2 axis drives the de-compensation of right ventricular function in pulmonary hypertension. Circ Res. 2015;116(1):56–69.
70. Potus F, Graydon C, Provencher S, Bonnet S. Vascular remodeling process in pulmonary arterial hypertension, with focus on miR-204 and miR-126 (2013 Grover conference series). Pulm Circ. 2014;4(2):175–84.
71. Potus F, Malenfant S, Graydon C, Mainguy V, Tremblay E, Breuils-Bonnet S, et al. Impaired angiogenesis and peripheral muscle microcirculation loss contribute to exercise intolerance in pulmonary arterial hypertension. Am J Respir Crit Care Med. 2014;190(3):318–28.
72. Potus F, Ruffenach G, Dahou A, et al. Downregulation of miR-126 contributes to the Failing right ventricle in pulmonary arterial hypertension. circulation 2015;132:932–43. doi:10.1161/CIRCULATIONAHA.115.016382.
73. Wei C, Henderson H, Spradley C, Li L, Kim I-K, Kumar S, et al. Circulating miRNAs as potential marker for pulmonary hypertension. PLoS One. 2013;8(5), e64396.

Chapter 13
microRNAs in Diabetic Kidney Disease

Arthur C.K. Chung

Abstract Diabetes and diabetic kidney diseases have continually exerted a great burden on our society. Although the recent advances in medical research have led to a much better understanding of diabetic kidney diseases, there is still no successful strategy for effective treatments for diabetic kidney diseases. Recently, treatment of diabetic kidney diseases relies either on drugs that reduce the progression of renal injury or on renal replacement therapies, such as dialysis and kidney transplantation. On the other hand, searching for biomarkers for early diagnosis and effective therapy is also urgent. Discovery of microRNAs has opened to a novel field for post-transcriptional regulation of gene expression. Results from cell culture experiments, experimental animal models, and patients under diabetic conditions reveal the critical role of microRNAs during the progression of diabetic kidney diseases. Functional studies demonstrate not only the capability of microRNAs to regulate expression of target genes, but also their therapeutic potential to diabetic kidney diseases. The existence of microRNAs in plasma, serum, and urine suggests their possibility to be biomarkers in diabetic kidney diseases. Thus, identification of the functional role of microRNAs provides an essentially clinical impact in terms of prevention and treatment of progression in diabetic kidney diseases as it enables us to develop novel, specific therapies and diagnostic tools for diabetic kidney diseases.

Keywords microRNAs • Diabetic kidney disease • Biomarker • Therapy

Introduction

As a chronic metabolic disease, diabetes occurs after our body is unable to generate sufficient insulin or our cells are unable to respond to the insulin produced. The global prevalence of diabetes is increasing and is predicted to reach the distressing

A.C.K. Chung, Ph.D. (✉)
Partner State Key Laboratory of Environmental and Biological Analysis, and Department of Chemistry, The Hong Kong Baptist University,
224, Waterloo Road, Kowloon Tong, Hong Kong, China

HKBU Institute for Research and Continuing Education, Shenzhen, China
e-mail: chungack@hkbu.edu.hk

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_13

number of 591.9 million by the year 2035 from 381.8 million in 2013 [1, 2]. It will be about 55 % increase within 20 years and this significant increase is attributed to an escalating tendency towards sedentary lifestyle and westernized choice of diet, leading to obesity. Diabetes is an important public health concern because the burden of diabetes is enormous which will provoke 5.1 million deaths and take up some US$548 billion dollars in health spending (11 % of the total spent worldwide) in 2013 [1].

Diabetes complications are divided into microvascular, which is due to damage to small blood vessels, and macrovascular, which is due to damage to larger blood vessels [3]. Microvascular complications include damage to eyes (retinopathy) leading to blindness, to kidneys (nephropathy) leading to renal failure, and to nerves (neuropathy) leading to impotence and diabetic foot disorders (which include severe infections leading to amputation). Among these complication, diabetic nephropathy (DN) draws much attention as it is the leading cause of chronic kidney disease in patients starting renal replacement therapy and is associated with increased cardiovascular mortality.

Although type 1 and type 2 diabetes are different in etiology and pathogenesis and different morphological changes of renal injury in type 1 and type 2 diabetic patients [4], patients with either type 1 or type 2 diabetes have comparable risks of renal injury in diseased kidney [5]. Diabetic nephropathy is characterized by the effacement of podocyte foot processes, gradual mesangial cell (MC) proliferation and hypertrophy, excessive accumulation of extracellular matrix (ECM) proteins, mesangial expansion, and thickening of the glomerular basement membrane (GBM) [3, 6]. Eventually these events lead to nodular glomerulosclerosis (Kimmelstiel-Wilson lesions) [7]. Tubular hypertrophy, thickening of the tubular basement membrane (TBM) and interstitial fibrosis occur in the tubulointerstitial area [7].

Diabetic nephropathy is a clinical syndrome characterized by persistent albuminuria (>300 mg/day or >200 μg/min) that is confirmed on at least two occasions 3–6 months apart, progressive decline in the glomerular filtration rate (GFR), and elevated arterial blood pressure. Present interventions of diabetic nephropathy consisting of rigorous glycemic control, antihypertensive therapy, such as angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs), are the first-line drugs. However, ACEIs and ARBs have been shown to slow down the progression of kidney disease but they are unable to halt this progression [8–12]. Therefore, there is an urgent need to search for effective therapy to halt the progression to the end-stage renal disease (ESRD) after the nephropathy has been established [13].

Furthermore, diabetic nephropathy is often related to other macrovascular diseases, such as cardiovascular disease. In the USA, diabetic nephropathy is contributed to almost 50 % of all ESRD [7]. Although dialysis or kidney transplantation regulate uremia and other symptoms of renal failure in patients with ESRD, the prognosis for patients with ESRD due to diabetes is not enthusiastic because more than 50 % of patients die within 5 years after diagnosis [14] and the 5-year survival rates is similar to that among patients with metastasized gastrointestinal carcinoma [13]. The increasing *population* with diagnosed *diabetes* and irreversible renal injury after onset of diabetic nephropathy demands the development of effective therapy to combat diabetic nephropathy in near future.

microRNAs in the Diabetic Kidney Disease

microRNAs, short noncoding RNAs with 22–25 nucleotides long, is able bind to the 3′ untranslated region (3′ UTR) of its target messenger RNA (mRNA) by imperfect complementary manner, leading to posttranscriptional gene silencing. Consequently microRNAs suppress gene expression via mRNA degradation, translation inhibition, or transcriptional inhibition [15, 16]. After two decades of the research, microRNAs are found to be essential in many cellular and biologic processes, such as proliferation, differentiation, and development, and in the regulation of genes related to immune responses, cancer, and insulin secretion [17–20]. Owing to the vital role in regulating gene expression, aberrant expression of microRNAs is found to be present in human diseases including cancer, hepatitis, and diabetes [18, 21–24]. More evidence demonstrates that microRNAs play a key role in renal field. As some reviews of microRNAs research on kidney development, function, and diseases have been previously published [25–40], this chapter will focus on the current research progress of microRNAs in diabetic nephropathy.

Glomerulosclerosis and interstitial fibrosis are two major pathological features during the progressive diabetic nephropathy. Damage of MCs and tubular epithelial cells (TECs) is attributed to fibrosis during diabetic nephropathy. All these events are resulted from aberrant glucose metabolism because this aberration will generate various harmful by-products such as advanced glycation end products (AGE), elevation of reactive oxygen species (ROS), and activation of protein kinase C (PKC). These events finally activate TGF-β signaling which stimulates renal fibrosis. Therefore, hyperglycemia acts as a trigger to induce the cascade of renal injury.

During the biosynthetic process of microRNAs, Dicer participates in both microRNAs biogenesis and microRNAs-mediated gene silencing [41–44]. Podocytes in the glomerular basement membrane are important to maintain the glomerular filtration barrier. Any podocyte dysfunction will result in glomerular pathologies as in diabetic nephropathy (DN) or other types of glomerulonephritis [45]. Deletion of Dicer in mouse podocytes demonstrates a loss of microRNAs and an induction to proteinuria, podocyte injury, and renal fibrosis [46–48], suggesting the vital role of microRNAs in the podocytes to keep renal functions to be normal. Recent evidence demonstrates that microRNAs act as vital regulators of gene expression during diabetic nephropathy. The involvement of microRNAs in diabetic nephropathy is summarized in Tables 13.1 and 13.2.

miR-192

The first microRNA reported in diabetic nephropathy is miR-192 [49]. Within glomeruli isolated from streptozotocin (STZ)-injected diabetic mice as well as diabetic *db*/*db* mice, the elevation miR-192 expression levels are associated with increased TGF-β1 and collagen 1a2 (Col1a2) levels. This increase of renal miR-192 expression is also observed in *db*/*db* mice, type 2 diabetes rat, and whole blood

Table 13.1 Upregulated microRNAs in diabetic nephropathy

microRNA	In vivo (animal mode)	In vitro (cell type)	Target	References
miR-192	STZ induced DN mice, DN in *db*/*db* mice	MCs (human, rat, mouse)	ZEB1/2	[49–53]
miR-216a miR-217	STZ induced DN mice, DN in *db*/*db* mice	Primary MCs (mouse)	PTEN	[57]
miR-216a	STZ induced DN mice, DN in *db*/*db* mice	Primary MCs (mouse)	Ybx1	[58]
miR-21	OVE26 type 1 diabetic mice (12 weeks of age)	MCs (human and rat)	PTEN	[63]
	db/*db* mice (10 or 20 weeks of age)	PTEs (mouse)	Smad7, TIMP3	[64, 67]
	kk-ay DN mice (T2DM) (20 weeks of age)	MCs, TECs (rat)	MMP-9, TIMP1	[65, 67]
miR-215	*db*/*db* mice	Primary MCs (mouse)	CTNNBIP1	[51]
miR-377	Spontaneous [(NOD/Lt) mice] and STZ induced DN mice	MCs (human and mouse)	PAK1 and MnSOD	[54]
miR-195	STZ induced DN mice	MCs (human and mouse)	BCLC2	[98, 99]

DN diabetic nephropathy, *MC* mesangial cells, *PTE* proximal tubule epithelial cells, *STZ* streptozotocin, *TEC* tubular epithelial cells

Table 13.2 Downregulated microRNAs in diabetic nephropathy

microRNA	In vitro (cell type)	In vivo (animal mode)	Target	References
miR-192	PTE human (HK-2 cells)	Patients with established DN	Zeb2	[61, 62]
miR-200a	TEC (rat)	STZ induced DN in apolipoprotein E knockout mice	TGF-β2	[89]
miR-29a	PTE human (HK-2 cells)		Col IVA1 & Col IVA2	[78]
miR-25	MCs (rat)	STZ induced DN rat	Nox4	[95]
miR-451	Primary MCs (mouse)	Early DN (*db*/*db* mice)	Ywhaz	[96]
miR-93	Renal microvascular	*db*/*db* mice	VEGF-A	[97]
	Endothelial cell			
	Mouse podocytes			

DN diabetic nephropathy, *MC* mesangial cells, *PTE* proximal tubule epithelial cells, *STZ* streptozotocin, *TEC* tubular epithelial cells

samples of type 2 diabetes patients [50–53]. Furthermore, TGF-β1 treatment increases miR-192 levels in both mouse MCs and isolated glomeruli from both type 1 and type 2 mouse models of diabetes [49, 53]. In vitro studies also demonstrate that miR-192 levels are increased in MCs and TECs after treatment with high

glucose, AGE, and TGF-β1 [54–56]. These results show the strong correlation between elevation of renal miR-192 levels and diabetic condition.

Several possible mechanisms have been purposed for the mediation of miR-192 during renal fibrosis. Firstly, miR-192 suppresses the E-box repressor Smad-1 interacting protein (Sip-1, Zeb2). As Sip-1 binds E-box enhancer elements in the Col1a2 gene, elevation of miR-192 then promotes collagen deposition in response to TGF-β1 [49]. Furthermore, miR-192 increases expression of miR-216a and miR-217 which target PTEN [57]. Thus, miR-192 can activate Akt kinase to promote fibrosis as Akt activation in mouse MCs induces signatures of diabetic nephropathy, such as ECM gene expression, apoptosis inhibition, and hypertrophy [57]. In addition, miR-216a is also reported to target Ybx1, an RNA binding protein and a component of P-bodies, and Ybx1 participates in TGF-β-induced collagen expression in mouse MCs [58].

The results from miR-192 knockout (KO) mice provide further support to the pathological role of miR-192 in diabetic nephropathy [59]. When compared to diabetic wild-type mice, deletion of miR-192 gene in type I diabetic mice decreases proteinuria, renal fibrosis, and hypertrophy [59]. Taken together, these studies show a pathological role of miR-192 in diabetic nephropathy.

The results from animal models show a pro-fibrotic role of miR-192 in diabetic nephropathy [49, 59, 60]. The results are opposite in human nephropathy [61, 62]. Reduced expression of miR-192 is associated with tubulointerstitial fibrosis and low estimated glomerular filtration rate (GFR) in patients with established diabetic nephropathy [61]. In a human proximal TEC line, HK-2 cells, treatment with TGF-β1 (10 ng/mL for 96 h) decreases the expression of miR-192, zinc finger E-box binding homeobox 1 (Zeb1), and E-cadherin but increases zinc finger E-box binding homeobox 2 (Zeb2), PAI-I, and vimentin [61]. In another report, treatment of TGF-β1 (10 ng/mL for 72 h) reduces levels of miR-192 and miR-215 in primary rat MCs and TEC, and in the renal cortex of apolipoprotein E knockout mice at 10 weeks of diabetes [62]. These differences of observations in murine models may be attributed to the different models and time points used [57, 62]. Reduced levels of miR-192 and miR-215, which target Zeb2, participate in TGF-β/CTGF-mediated changes in E-cadherin expression. These results demonstrate that miR-192/215 may not affect fibrosis by directly changing the levels of fibrotic markers and ECM proteins [62]. Taken together, the different findings in levels of miR-192 in human and animal models of diabetic nephropathy suggest that signaling mechanism during diabetic kidney injury is complicated.

miR-21

The pro-fibrotic role of miR-21 is more obvious as results of in vitro studies show that miR-21 abundance is increased in TECs and MCs after treatment with TGF-β1 or under diabetic condition [55, 63–66]. Higher levels of miR-21 in renal cortices are found in type 1 (OVE26) and type 2 (*kk-ay* and *db*/*db*) diabetic mouse models

[63–65, 67] although a report demonstrates a reduction of miR-21 expression during early DN in diabetic *db*/*db* mice [68]. In human, miR-21 abundance is increased in kidney biopsies from diabetic patients compared to healthy controls [67]. Furthermore, the pro-fibrotic role of miR-21 is further reinforced by the in vitro studies that miR-21 upregulates the expression of ECM and α-SMA in TECs and MCs after treatment of TGF-β1 or under diabetic condition [55, 63–66]. In addition, overexpression of miR-21 in kidney cells also enhances but suppression of miR-21 inhibits renal inflammation under diabetic condition [64].

Several possible mechanisms have been purposed for the mediation of miR-21 during renal injury and these mechanisms may be related to putative target genes of miR-21 and the activation of TGF-β signaling during diabetic condition [69, 70]. The first evidence is that miR-21 may activate the TGF-β canonical signaling by inhibiting Smad7, which prevents activation of TGF-β signaling [64]. In addition, miR-21 may mediate the TGF-β noncanonical signaling by targeting to Sprouty (SPRY) as SPRY, a potent inhibitor of Ras/MEK/ERK, suppresses TGF-β-dependent fibrogenic activities [71]. As phosphatase and tensin homolog (PTEN) may be one of targets of miR-21 [72, 73], activation of Akt pathway by suppressing PTEM via miR-21 may be another mechanism for miR-21 to be involved in diabetic kidney injury. Suppression of PTEN by miR-21 also increases activities of phosphatidylinositide 3-kinases (PI3K) and Akt, and subsequently activates metalloproteinase-2 (MMP-2) expression [72]. Recently, miR-21 is found to mediate the reciprocal regulation of PTEN levels and AKT1 substrate 1 (PRAS40), a negative regulator of Tor complex 1 (TORC1) activity during diabetic nephropathy [63]. As both metalloproteinases and tissue inhibitors of metalloproteinases (TIMPs) activities play a key role to regulate ECM turnover during fibrosis, the recent findings of TIMP3 as a potential miR-21 target suggest that miR-21 mediate several pathways to enhance renal injury during diabetic nephropathy [67].

miR-29

As miR-192 and miR-21 are pro-fibrotic microRNAs, miR-29s are anti-fibrotic microRNAs. Normally abundance of miR-29 family (miR-29a, miR-29b, and miR-29c) is always high in kidney, lung, and heart [74]. During fibrotic diseases, its abundance significantly decreases in animal models and human samples of heart, lung, and kidney [75–77]. Furthermore, treatment with TGF-β1 or under diabetic condition downregulates the abundance of the miR-29 family in cultured MCs, TECs, and podocytes but upregulates the abundance of ECM proteins [55, 75, 78, 79], suggesting a protective role of miR-29 during renal injury.

Its protective role against fibrosis has been shown in various fibrotic disease models. Overexpression of miR-29 inhibits but knockdown of miR-29 enhances abundance of fibrotic markers in cell lines from heart, lung, and kidney after TGF-β treatment [71, 75, 76, 79–82]. More importantly, delivery of miR-29b either before or after established obstructive nephropathy successfully halts progressive renal

fibrosis in a mouse model of unilateral ureteral obstruction nephropathy [75], supporting the anti-fibrotic properties of miR-29.

By employing different disease models of heart, lung, and kidneys, miR-29 suppresses fibrosis mainly by inhibiting the ECM-related gene transcription because more than 20 different ECM-related genes have been validated to be miR-29 targets via reporter gene assays and some of them are induced by TGF-β signaling [76, 81, 83].

The anti-fibrotic properties of miR-29 are also confirmed in diabetic condition that inhibition of miR-29a increased the risk of excess collagen deposition [78]. Furthermore, miR-29a downregulates collagen IV (Col IV) expression by directly targeting the 3′ UTRs of the collagen genes Col IV a1 and Col IV a2 in HK-2 cells [78].

miR-200

The ability to maintain epithelial differentiation is the well-established function of miR-200 family (miR-200a, miR-200b, miR-200c, miR-429, and miR-141). Thus it is believed that miR-200 family inhibits fibrosis via this ability [84–86]. Treatment with TGF-β reduces the abundance of these microRNAs and the cells will undergo epithelial to mesenchymal transition (EMT) [85–88], suggesting that TGF-β may mediate these microRNAs to promote EMT. Although it is believed that proximal TECs may undergo EMT to induce renal fibrosis [89], this notion that EMT contributes to renal fibrosis has been lately confronted. More studies should be done to clarify the role of miR-200 in kidney diseases.

In renal TECs, both TGF-β1 and TGF-β2 can reduce abundance of the miR-200 family in a Smad signaling dependent manner [89, 90]. Similarly, reduction of renal miR-200a and miR-141 occurs in kidneys of a mouse model of diabetic nephropathy [89], suggesting a protective role of miR-200 family in diabetic nephropathy.

However, elevation of miR-200b/c levels is found in glomeruli from type 1 (streptozotocin) and type 2 (*db*/*db*) diabetic mice, and in mouse mesangial cells treated with TGF-β1 in vitro [53]. Recently, activation of miR-200b and miR-200c and their target FOG2, an inhibitor of phosphatidylinositol 3-kinase activation, is shown to be a possible way of how TGF-β induces Akt signaling in glomerular mesangial cells [91]. The reduction of FOG2 expression is observed in the glomeruli of diabetic mice and TGF-β-treated mouse MC. It is unexpectedly that increase of miR-200b/c levels is detected in diabetic mouse glomeruli and TGF-β-treated MC [91]. Transfection with miR-200b/c mimics in MC considerably reduces FOG2 expression and increases cell hypertrophy which is confirmed by FOG2 knockdown in MC. In addition, suppression of FOG2 by miR-200b/c also activates ERKs, which is through PI3K activation [91]. These new findings suggest a new mechanism for TGF-β-induced Akt activation through FOG2 suppression by miR-200b/c, which results in glomerular mesangial hypertrophy during diabetic kidney disease. However, the differences of miR-200 expression in diabetic kidneys are possibly due to the differences in the origin of cell lines examined, the treatments performed, and the use of different animal models between studies.

microRNAs in Glomerular Permeability and Podocytes

Podocytes, which are epithelial cell of the visceral layer of a renal glomerulus, are important to retain glomerular selectivity and control the ECM synthesis in glomerular basement membrane (GBM) [6]. During diabetic kidney disease, impairment of glomerular podocytes, deposition of extracellular ECM in glomeruli, and hypertrophy and expansion in the glomerular mesangium are observed within the diseased kidney [45]. As a result, proteinuria will occur and kidney function will decline.

Role of microRNAs in podocyte function is demonstrated from studies from two independent lines of Dicer KO mice generated for podocytes [46, 48]. It is found that proteinuria is observed in KO mice in 3 weeks after birth and rapidly develops into end-stage kidney disease. In addition, various abnormalities are found in glomeruli of KO mice, such as podocyte apoptosis and depletion, irregular and split areas of the glomerular basement membrane, foot process effacement, mesangial expansion, capillary dilation, and glomerulosclerosis [48], suggesting that proper function of Dicers to produce microRNAs in podocytes is required.

A recent study of specific deletion of Dicer in mouse podocyte discloses an enrichment of predicted miR-30 target genes among the upregulated genes [92]. Abundance of miR-30s is high in glomerular podocytes in mice and TGF-β inhibits miR-30s levels in podocytes [92]. When diabetic condition upregulates TGF-β levels and activates TGF-β signaling, TGF-β-suppression of miR-30s in podocyte may contribute to TGF-β-induced apoptosis in podocytes.

During diabetic kidney diseases, urinary podocyte excretion is observed and this feature is associated with the decreased adhesive capacity after damage of podocyte [93]. Recently, miR-124 levels are found to impair proper podocytic adhesive capacity [94]. During diabetic kidney disease, miR-124 abundance in kidneys is elevated in STZ-induced uninephrectomized diabetic rats and inhibition of renal miR-124 improves podocytic adhesive capacity [94].

Other microRNAs

Here are other microRNAs involved in diabetic kidney disease. Further studies on these microRNAs may shed more light on their roles in diabetic nephropathy.

Abundance of miR-25 is reduced by high glucose in MCs or in rat diabetic kidney. As it targets NADPH oxidase 4 (Nox4), this reduction results in an increase of Nox4 expression and ROS production [95], suggesting that miR-25 may protect the kidney from oxidative stress.

miR-215 may be involved in Wnt/β-catenin signaling as it targets catenin-beta interacting protein 1, which suppressed Wnt/β-catenin signaling. In addition, miR-215 is significantly elevated under diabetic conditions both in vitro (MCs) and in vivo (*db*/*db* mice) [51] and miR-215 mediates TGF-β1-induced fibronectin levels via activating β-catenin [51].

Renal miR-451 is reduced during early diabetic nephropathy in *db*/*db* mice. miR-451 controls p38 mitogen-activated protein kinases (MAPK) signaling by targeting of Ywhaz (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta) [96], suggesting that miR-451 protects mesangial hypertrophy.

Abundance of miR-377 is elevated in high glucose cultured or TGF-β treated human and mouse MCs, and in kidney of spontaneous (NOD/Lt) and STZ-induced type 1 diabetes mouse model [54]. It may participate in fibronectin production and oxidative stress during diabetic nephropathy. miR-377 targets p21-activated kinase-1 (PAK1) and manganese superoxide dismutase (MnSOD), which enhance fibronectin expression [54].

As miR-93 is primarily expressed in glomeruli and TEC, it is one of signature microRNAs in hyperglycemic conditions [97]. However, abundance of miR-93 is reduced in cultured podocyte cells, renal microvascular endothelial cells, and glomeruli of diabetic mice [97]. High levels of miR-93 decreases expression of vascular endothelial growth factor (VEGF), a predicted target of miR-93 [97]. As VEGF targets collagen IV and fibronectin, the repression of miR-93 during diabetic nephropathy may contribute to the production of collagen and fibronectin.

High abundance of miR-195 was found in both STZ-induced type 1 diabetic mice and MCs and podocytes cultured in high glucose. Overexpression of miR-195 reduces B-cell lymphoma 2 protein levels but increases caspase-3 in podocytes and MCs, suggesting that miR-195 may mediate apoptosis in diabetic kidney disease [98, 99].

Overall, the aberrant expression of these microRNAs in pathogenesis elicits the critical role of microRNAs in diabetic nephropathy.

Regulations of microRNA During Diabetic Kidney Disease

It is still unknown what exact mechanism of how high glucose condition controls microRNA abundance during diabetic kidney disease. It is generally accepted that TGF-β signaling may be the major signaling pathway to enhance microRNA biogenesis during diabetic condition [40]. TGF-β signaling has been reported to promote the conversion of primary transcripts of some microRNAs into their mature form by the Drosha complex [100]. For example, Smad3, the downstream mediator of TGF-β signaling, physically interacts with Drosha to catalyze the process from pri-miR-21 to mature miR-21.

We also show that the transcription of miR-21, miR-192, miR-433, and the miR-29 family during renal diseases is regulated by TGF-β/Smad3 signaling [56, 66, 75, 101, 102]. TGF-β suppresses miR-29 expression but induces miR-2, miR-433, and miR-192 expression. This regulation depended on the presence of Smad3 as shown in Smad2 or Smad3 KO mouse embryonic fibroblasts (MEF), and in MCs and TECs knocking down for Smad2 or Smad3, or overexpressing Smad7 [55, 56, 66, 75]. Furthermore, Smad3 also physically interacts with Smad binding site (SBE) located in their promoters to regulate the expression of these microRNAs [56, 66, 75, 101].

Smad7, an inhibitory Smad, protects kidneys from fibrosis not only by suppressing Smad3 activation, but also by regulating TGF-β/Smad3-mediated microRNAs via maintaining renal miR-29b but suppressing miR-192 and miR-21 [55, 56, 103]. This notion has been confirmed by the results from several mouse models of kidney diseases induced in mice lacking Smad3, Smad7, or having conditional knockout (KO) for Smad2 or overexpressing renal Smad7 [55, 56, 66, 75, 101].

A recent study further extends the regulation of microRNAs in diabetic kidney disease to p53. It shows that TGF-β induces a crosstalk between p53 and miR-192 [59]. Abundance of TGF-β1, p53, and miR-192 is known to be increased in diseased glomeruli of diabetic mice [59, 60, 104].

When TGF-β-induced miR-192 abundance is reduced in vivo, this reduction will then reduce renal p53 expression in streptozotocin induced diabetic mice [59]. This correlation between miR-192 and renal expression of TGF-β and p53 is further supported by the results from miR-192 KO type I diabetic mice [59]. Taken together, the TGF-β/Smad3 signaling is important to control microRNA abundance during diabetic nephropathy.

Therapeutic Potential of microRNAs in Diabetic Kidney Disease

Besides searching the functions of microRNAs, the focus has recently shifted to the therapeutic potential of the microRNAs and ability to be a biomarker in diabetic nephropathy.

Many microRNA studies have shown that restoring or inhibiting the abundance of microRNAs in kidney cells under diabetic condition is able to reduce the levels of ECM [64, 65, 96]. In addition, delivery of microRNA mimics, inhibitors, or plasmids for overexpressing or knocking down microRNAs in rodent experimental models provides solid evidences that regulating microRNA abundance has therapeutic potential to cure diabetic nephropathy. For example, the in vivo transfer of antagomiR-21 ameliorates creatinine clearance ratio and urine albumin creatinine ratio, and reduces the abundance of tissue inhibitor of metalloproteinase 1 (TIMP-1), Col IV, and fibronectin in kidney of diabetic mice [65]. Delivery of *miR-21* knockdown plasmids into the diabetic kidneys of *db*/*db* mice at age 10 weeks considerably ameliorates both microalbuminuria and renal fibrosis and inflammation at age 20 weeks [64]. Reducing miR-29c by an antisense oligonucleotide restores expression of Sprouty-1 and ameliorates albuminuria and mesangial matrix expansion in the type 2 (*db*/*db*) diabetic mice [50]. Inhibition of miR-192 with modified anti-sense oligonucleotides significantly attenuates proteinuria in mice with diabetic nephropathy and suppresses oxidative stress and the renal fibrosis and hypertrophy [60]. Knockdown of miR-215 with antagomiR-215 restores CTNNBIP expression and inhibits Wnt/β-catenin signaling and expression of α-SMA and fibronectin in the *db*/*db* mouse kidney [51]. Overexpression of miR-451 inhibits

glomerular MC proliferation in vivo [96]. These successful results from rodent diabetic nephropathy models demonstrate two important aspects.

Firstly, altering microRNA activity in diabetic kidneys can hold the progression of diabetic nephropathy which is not shown in current drug treatment. Theses promising results from experimental models demonstrate the possibility of applying microRNA therapy in the clinical practice. In addition to the conventional plasmid delivery, recent development of chemically modified oligonucleotides that are stable in the circulation, can freely enter cells to bind to specific microRNA and silence it [31], provides possible and effective delivery methods to ensure the success of microRNA therapy in renal diseases. However, there are still some obstacles for microRNA therapy. Further attention may focus on the risk of off-target effects of microRNAs, specificity of delivery methods, and nonspecific immune response. Therefore, it is still a long way for clinic application with microRNA-based therapy against diabetic nephropathy.

On the other hand, circulating microRNAs in serum, plasma, and urine have been used as biomarkers for many diseases to reflect a response to the pathophysiological stresses [105–108]. The possibility to use circulating microRNAs as biomarkers in diabetic nephropathy is investigating because the variations of microRNA levels in the body fluids from patients with diabetic nephropathy may reveal the progression of the disease.

A recent study shows that a total of 27 microRNAs at significantly different levels are found in urine from the patients at different stages of diabetic nephropathy [109]. In addition, these 27 microRNAs have previously been found to participate in signaling pathways of renal fibrosis during diabetic kidney disease. An another study of microRNA abundance in urinary exosomes from type 1 diabetic patients with and without incipient diabetic nephropathy shows that miR-130a and miR-145 are higher but miR-155 and miR-424 are decreased in patients with microalbuminuria [110]. These results are supported by studies in cells and animal models. Abundance of miR-145 is induced after treatment with high glucose in both MCs and MC-derived exosomes. Similarly in an animal model of early experimental diabetic nephropathy, the elevation of urinary exosomal miR-145 levels is tightly correlated with miR-145 overexpression within the glomeruli [110]. These results propose a possible correlation between circulating microRNA and its renal expression during diabetic kidney disease.

As microRNAs found in circulation or urine may reflect the progression of diabetic nephropathy, the early detection of their presence in circulation or urine may enable us to predict the disease progression. To achieve this purpose, the threshold of detection of microRNAs by current amplification methods should be increased and a database of the correlation between patterns of microRNA released in circulation or urine and specific microRNA expression profiles in damaged kidneys should be established.

Conclusion

After the discovery of microRNAs and characterization of their functions in kidney diseases for two decades, more effort is still required to understand the specific role and mechanism of microRNAs in renal pathophysiology. Currently, it is known that specific microRNAs affect renal physiology by altering transcript levels of target genes, mediating TGF-β actions on renal fibrosis, disturbing normal functions of MC, TEC, and podocyte during renal diseases [25, 29, 34, 35].

However, the exact mechanism of how microRNAs regulate the diabetic renal injury is still unclear. In principle, one microRNA is able to regulate multiple target genes. When the number of report of certain microRNA is increasing, the number of target genes is also increasing. For example, more than eight direct targets of miR-21 and more than ten targets of miR-29 are found in renal research so far. This target search only gives us confusion because we do not know the relationship among these possible mechanisms. In addition, the report about the relationship among the target genes of a specific microRNA is scarce and it is still an open field for future microRNA research. Hopefully, the rapid development of high-throughput validation and proteomic analysis can help us to identify real microRNA targets and determine the exact mechanism of microRNAs in renal diseases in near future.

Finally, microRNAs act as essential regulators of gene expression during diabetic nephropathy. The further understanding of their roles during diabetic kidney disease offers us not only an alternative to halt disease progression, but also putative biomarkers for early prediction of kidney diseases.

Acknowledgements This work was supported by grants from National Natural Science Foundation of China (General Program 81170681 and 21477101); the Research Grant Council of Hong Kong (RGC GRF 463612, 464010, 763908, 764109, and 14104314); Faculty Research Grant from the Hong Kong Baptist University (30-13-170FRG1/13-14/070); R&D funding of basic research, Shenzhen (General Program JC201105201059A), and national and provincial funding from Shenzhen City (GJHS20120702105523297). The author declared no competing interests.

References

1. Federation ID. IDF diabetes atlas. Brussels: IDF; 2013.
2. Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract. 2010;87(1):4–14.
3. Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev. 2013;93(1): 137–88.
4. Fioretto P, Caramori ML, Mauer M. The kidney in diabetes: dynamic pathways of injury and repair. The Camillo Golgi lecture 2007. Diabetologia. 2008;51(8):1347–55.
5. Hasslacher C, Ritz E, Wahl P, Michael C. Similar risks of nephropathy in patients with type I or type II diabetes mellitus. Nephrol Dial Transplant. 1989;4(10):859–63.
6. Miner JH. Renal basement membrane components. Kidney Int. 1999;56(6):2016–24.
7. Kanwar YS, Sun L, Xie P, Liu FY, Chen SA. Glimpse of various pathogenetic mechanisms of diabetic nephropathy. Annu Rev Pathol. 2011;6(1):395–423.

8. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD. The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. N Engl J Med. 1993;329(20):1456–62.
9. Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, Parving H-H, et al. Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. N Engl J Med. 2001;345(12):861–9.
10. Lewis EJ, Hunsicker LG, Clarke WR, Berl T, Pohl MA, Lewis JB, et al. Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. N Engl J Med. 2001;345(12):851–60.
11. Retinopathy E. Nephropathy in patients with type 1 diabetes four years after a trial of intensive therapy. The diabetes control and complications trial/epidemiology of diabetes interventions and complications research group. [Erratum appears in N Engl J Med 2000 May 4; 342 (18): 1376]. N Engl J Med. 2000;342(6):381–90.
12. Ziyadeh FN, Sharma K. Overview: combating diabetic nephropathy. J Am Soc Nephrol. 2003;14(5):1355–7.
13. Ritz E, Orth SR. Nephropathy in patients with type 2 diabetes mellitus. N Engl J Med. 1999;341(15):1127–33.
14. Blumenthal SS. Evolution of treatment for diabetic nephropathy: historical progression from RAAS inhibition and onward. Postgrad Med. 2011;123(6):166–79.
15. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75(5):843–54.
16. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell. 1993;75(5):855–62.
17. Taganov KD, Boldin MP, Chang K-J, Baltimore D. NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci. 2006;103(33):12481–6.
18. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. Nat Rev Cancer. 2006;6(4):259–69.
19. Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, MacDonald PE, et al. A pancreatic islet-specific microRNA regulates insulin secretion. Nature. 2004;432(7014):226–30.
20. Bushati N, Cohen SM. microRNA functions. Annu Rev Cell Dev Biol. 2007;23:175–205.
21. Janssen HLA, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, et al. Treatment of HCV infection by targeting microRNA. N Engl J Med. 2013;368(18): 1685–94.
22. Chang T-C, Mendell JT. MicroRNAs in vertebrate physiology and human disease. Annu Rev Genomics Hum Genet. 2007;8:215–39.
23. Chen C. MicroRNAs as oncogenes and tumor suppressors. N Engl J Med. 2005;353(17):1768.
24. Hennessy E, O'Driscoll L. Molecular medicine of microRNAs: structure, function and implications for diabetes. Expert Rev Mol Med. 2008;10:e24.
25. Saal S, Harvey SJ. MicroRNAs and the kidney: coming of age. Curr Opin Nephrol Hypertens. 2009;18(4):317–23.
26. Kato M, Natarajan R. MicroRNA cascade in diabetic kidney disease: big impact initiated by a small RNA. Cell Cycle. 2009;8(22):3613–4.
27. Kato M, Arce L, Natarajan R. MicroRNAs and their role in progressive kidney diseases. Clin J Am Soc Nephrol. 2009;4(7):1255–66.
28. Kaucsár T, Rácz Z, Hamar P. Post-transcriptional gene-expression regulation by micro RNA (miRNA) network in renal disease. Adv Drug Deliv Rev. 2010;62(14):1390–401.
29. Li JY, Yong TY, Michael MZ, Gleadle JM. Review: the role of microRNAs in kidney disease. Nephrology (Carlton). 2010;15(6):599–608.
30. Akkina S, Becker BN. MicroRNAs in kidney function and disease. Transl Res. 2011; 157(4):236–40.
31. Lorenzen JM, Haller H, Thum T. MicroRNAs as mediators and therapeutic targets in chronic kidney disease. Nat Rev Nephrol. 2011;7(5):286–94.
32. Bhatt K, Mi Q-S, Dong Z. MicroRNAs in kidneys: biogenesis, regulation, and pathophysiological roles. Am J Physiol Renal Physiol. 2011;300(3):F602–10.

33. Ho J, Kreidberg JA. The long and short of microRNAs in the kidney. J Am Soc Nephrol. 2012;23(3):400–4.
34. Kantharidis P, Wang B, Carew RM, Lan HY. Diabetes complications: the microRNA perspective. Diabetes. 2011;60(7):1832–7.
35. Kato M, Park JT, Natarajan R. MicroRNAs and the glomerulus. Exp Cell Res. 2012;318(9):993–1000.
36. Karthikeyan Chandrasekaran DSK, Sepramaniam S, Armugam A, Wintour EM, Bertram JF, Jeyaseelan K. Role of microRNAs in kidney homeostasis and disease. Kidney Int. 2012; 81(7):617–27.
37. Pandey AK, Agarwal P, Kaur K, Datta M. MicroRNAs in diabetes: tiny players in big disease. Cell Physiol Biochem. 2009;23(4–6):221–32.
38. Ma L, Qu L. The function of microRNAs in renal development and pathophysiology. J Genet Genomics. 2013;40(4):143–52.
39. Ho J, Kreidberg JA. MicroRNAs in renal development. Pediatr Nephrol. 2013;28(2): 219–25.
40. Chung AC, Yu X, Lan HY. MicroRNA and nephropathy: emerging concepts. Int J Nephrol Renovascu Dis. 2013;6:169–79.
41. Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, et al. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature. 2005;436(7051):740–4.
42. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell. 2005;123(4):631.
43. MacRae IJ, Ma E, Zhou M, Robinson CV, Doudna JA. In vitro reconstitution of the human RISC-loading complex. Proc Natl Acad Sci. 2008;105(2):512–7.
44. Robb GB, Rana TM. RNA helicase A interacts with RISC in human cells and functions in RISC loading. Mol Cell. 2007;26(4):523–37.
45. Vallon V, Komers R. Pathophysiology of the diabetic kidney. Compr Physiol. 2011;1(3): 1175–232.
46. Harvey SJ, Jarad G, Cunningham J, Goldberg S, Schermer B, Harfe BD, et al. Podocyte-specific deletion of dicer alters cytoskeletal dynamics and causes glomerular disease. J Am Soc Nephrol. 2008;19(11):2150–8.
47. Ho J, Ng KH, Rosen S, Dostal A, Gregory RI, Kreidberg JA. Podocyte-specific loss of functional microRNAs leads to rapid glomerular and tubular injury. J Am Soc Nephrol. 2008;19(11):2069–75.
48. Shi S, Yu L, Chiu C, Sun Y, Chen J, Khitrov G, et al. Podocyte-selective deletion of dicer induces proteinuria and glomerulosclerosis. J Am Soc Nephrol. 2008;19(11):2159–69.
49. Kato M, Zhang J, Wang M, Lanting L, Yuan H, Rossi JJ, et al. MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-β-induced collagen expression via inhibition of E-box repressors. Proc Natl Acad Sci. 2007;104(9):3432–7.
50. Long J, Wang Y, Wang W, Chang BH, Danesh FR. MicroRNA-29c is a signature microRNA under high glucose conditions that targets sprouty homolog 1, and its in vivo knockdown prevents progression of diabetic nephropathy. J Biol Chem. 2011;286(13):11837–48.
51. Mu J, Pang Q, Guo Y-H, Chen J-G, Zeng W, Huang Y-J, et al. Functional implications of microRNA-215 in TGF-β1-induced phenotypic transition of mesangial cells by targeting CTNNBIP1. PLoS One. 2013;8(3):e58622.
52. Karolina DS, Armugam A, Tavintharan S, Wong MT, Lim SC, Sum CF, et al. MicroRNA 144 impairs insulin signaling by inhibiting the expression of insulin receptor substrate 1 in type 2 diabetes mellitus. PLoS One. 2011;6(8):e22839.
53. Kato M, Arce L, Wang M, Putta S, Lanting L, Natarajan R. A microRNA circuit mediates transforming growth factor-β1 autoregulation in renal glomerular mesangial cells. Kidney Int. 2011;80(4):358–68.
54. Wang Q, Wang Y, Minto AW, Wang J, Shi Q, Li X, et al. MicroRNA-377 is up-regulated and can lead to increased fibronectin production in diabetic nephropathy. FASEB J. 2008;22(12): 4126–35.

55. Chung AC, Dong Y, Yang W, Zhong X, Li R, Lan HY. Smad7 suppresses renal fibrosis via altering expression of TGF-beta/Smad3-regulated microRNAs. Mol Ther. 2013;21(2): 388–98.
56. Chung AC, Huang XR, Meng X, Lan HY. miR-192 mediates TGF-β/Smad3-driven renal fibrosis. J Am Soc Nephrol. 2010;21(8):1317–25.
57. Kato M, Putta S, Wang M, Yuan H, Lanting L, Nair I, et al. TGF-β activates Akt kinase through a microRNA-dependent amplifying circuit targeting PTEN. Nat Cell Biol. 2009;11(7):881–9.
58. Kato M, Wang L, Putta S, Wang M, Yuan H, Sun G, et al. Post-transcriptional up-regulation of Tsc-22 by Ybx1, a target of miR-216a, mediates TGF-β-induced collagen expression in kidney cells. J Biol Chem. 2010;285(44):34004–15.
59. Deshpande SD, Putta S, Wang M, Lai JY, Bitzer M, Nelson RG, et al. Transforming growth factor-beta induced cross talk between p53 and a microRNA in the pathogenesis of diabetic nephropathy. Diabetes. 2013;62(9):3151–62.
60. Putta S, Lanting L, Sun G, Lawson G, Kato M, Natarajan R. Inhibiting microRNA-192 ameliorates renal fibrosis in diabetic nephropathy. J Am Soc Nephrol. 2012;23(3):458–69.
61. Krupa A, Jenkins R, Luo DD, Lewis A, Phillips A, Fraser D. Loss of microRNA-192 promotes fibrogenesis in diabetic nephropathy. J Am Soc Nephrol. 2010;21(3):438–47.
62. Wang B, Herman-Edelstein M, Koh P, Burns W, Jandeleit-Dahm K, Watson A, et al. E-cadherin expression is regulated by miR-192/215 by a mechanism that is independent of the profibrotic effects of transforming growth factor-β. Diabetes. 2010;59(7):1794–802.
63. Dey N, Das F, Mariappan MM, Mandal CC, Ghosh-Choudhury N, Kasinath BS, et al. MicroRNA-21 orchestrates high glucose-induced signals to TOR complex 1, resulting in renal cell pathology in diabetes. J Biol Chem. 2011;286(29):25586–603.
64. Zhong X, Chung A, Chen H, Dong Y, Meng X, Li R, et al. miR-21 is a key therapeutic target for renal injury in a mouse model of type 2 diabetes. Diabetologia. 2013;56(3):663–74.
65. Wang J, Gao Y, Ma M, Li M, Zou D, Yang J, et al. Effect of miR-21 on renal fibrosis by regulating MMP-9 and TIMP1 in kk-ay diabetic nephropathy mice. Cell Biochem Biophys. 2013;67(2):537–46.
66. Zhong X, Chung ACK, Chen H-Y, Meng X-M, Lan HY. Smad3-mediated upregulation of miR-21 promotes renal fibrosis. J Am Soc Nephrol. 2011;22(9):1668–81.
67. Fiorentino L, Cavalera M, Mavilio M, Conserva F, Menghini R, Gesualdo L, et al. Regulation of TIMP3 in diabetic nephropathy: a role for microRNAs. Acta Diabetol. 2013;50(6): 965–9.
68. Zhang Z, Peng H, Chen J, Chen X, Han F, Xu X, et al. MicroRNA-21 protects from mesangial cell proliferation induced by diabetic nephropathy in db/db mice. FEBS Lett. 2009;583(12):2009–14.
69. Meng XM, Chung AC, Lan HY. Role of the TGF-beta/BMP-7/Smad pathways in renal diseases. Clin Sci. 2013;124(4):243–54.
70. Lan HY, Chung AC. TGF-beta/Smad signaling in kidney disease. Semin Nephrol. 2012;32(3): 236–43.
71. Ding Q, Gladson CL, Wu H, Hayasaka H, Olman MA. Focal adhesion kinase (FAK)-related non-kinase inhibits myofibroblast differentiation through differential MAPK activation in a FAK-dependent manner. J Biol Chem. 2008;283(40):26839–49.
72. Roy S, Khanna S, Hussain SR, Biswas S, Azad A, Rink C, et al. MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovasc Res. 2009;82(1):21–9.
73. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature. 2008;456(7224):980–4.
74. Kriegel AJ, Liu Y, Fang Y, Ding X, Liang M. The miR-29 family: genomics, cell biology, and relevance to renal and cardiovascular injury. Physiol Genomics. 2012;44(4):237–44.
75. Qin W, Chung ACK, Huang XR, Meng X-M, Hui DSC, Yu C-M, et al. TGF-β/Smad3 signaling promotes renal fibrosis by inhibiting miR-29. J Am Soc Nephrol. 2011;22(8):1462–74.

76. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, et al. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci U S A. 2008;105(35):13027–32.
77. Xiao J, Meng XM, Huang XR, Chung AC, Feng YL, Hui DS, et al. miR-29 inhibits bleomycin-induced pulmonary fibrosis in mice. Mol Ther. 2012;20(6):1251–60.
78. Du B, Ma L-M, Huang M-B, Zhou H, Huang H-L, Shao P, et al. High glucose down-regulates miR-29a to increase collagen IV production in HK-2 cells. FEBS Lett. 2010;584(4):811–6.
79. Wang B, Komers R, Carew R, Winbanks CE, Xu B, Herman-Edelstein M, et al. Suppression of microRNA-29 expression by TGF-beta1 promotes collagen expression and renal fibrosis. J Am Soc Nephrol. 2012;23(2):252–65.
80. Du T, Zamore PD. MicroPrimer: the biogenesis and function of microRNA. Development. 2005;132(21):4645–52.
81. Liu Y, Taylor NE, Lu L, Usa K, Cowley Jr AW, Ferreri NR, et al. Renal medullary microRNAs in Dahl salt-sensitive rats: miR-29b regulates several collagens and related genes. Hypertension. 2010;55(4):974–82.
82. Ye Y, Hu Z, Lin Y, Zhang C, Perez-Polo JR. Downregulation of microRNA-29 by antisense inhibitors and a PPAR-gamma agonist protects against myocardial ischaemia-reperfusion injury. Cardiovasc Res. 2010;87(3):535–44.
83. Cushing L, Kuang PP, Qian J, Shao F, Wu J, Little F, et al. miR-29 is a major regulator of genes associated with pulmonary fibrosis. Am J Respir Cell Mol Biol. 2011;45(2):287–94.
84. Howe EN, Cochrane DR, Richer JK. The miR-200 and miR-221/222 microRNA families: opposing effects on epithelial identity. J Mammary Gland Biol Neoplasia. 2012;17(1):65–77.
85. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008;10(5):593–601.
86. Korpal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J Biol Chem. 2008;283(22):14910–4.
87. Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev. 2008;22(7):894–907.
88. Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, et al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep. 2008;9(6):582–9.
89. Wang B, Koh P, Winbanks C, Coughlan MT, McClelland A, Watson A, et al. miR-200a prevents renal fibrogenesis through repression of TGF-β2 expression. Diabetes. 2011;60(1):280–7.
90. Xiong M, Jiang L, Zhou Y, Qiu W, Fang L, Tan R, et al. The miR-200 family regulates TGF-beta1-induced renal tubular epithelial to mesenchymal transition through Smad pathway by targeting ZEB1 and ZEB2 expression. Am J Physiol Renal Physiol. 2012;302(3):F369–79.
91. Park JT, Kato M, Yuan H, Castro N, Lanting L, Wang M, et al. FOG2 protein down-regulation by transforming growth factor-beta1-induced microRNA-200b/c leads to Akt kinase activation and glomerular mesangial hypertrophy related to diabetic nephropathy. J Biol Chem. 2013;288(31):22469–80.
92. Shi S, Yu L, Zhang T, Qi H, Xavier S, Ju W, et al. Smad2-dependent downregulation of miR-30 is required for TGF-beta-induced apoptosis in podocytes. PLoS One. 2013;8(9):e75572.
93. Vogelmann SU, Nelson WJ, Myers BD, Lemley KV. Urinary excretion of viable podocytes in health and renal disease. Am J Physiol Renal Physiol. 2003;285(1):F40–8.
94. Li D, Lu Z, Jia J, Zheng Z, Lin S. MiR-124 is related to podocytic adhesive capacity damage in STZ-induced uninephrectomized diabetic rats. Kidney Blood Press Res. 2013;37(4–5):422–31.
95. Fu Y, Zhang Y, Wang Z, Wang L, Wei X, Zhang B, et al. Regulation of NADPH oxidase activity is associated with miRNA-25-mediated NOX4 expression in experimental diabetic nephropathy. Am J Nephrol. 2010;32(6):581–9.

96. Zhang Z, Luo X, Ding S, Chen J, Chen T, Chen X, et al. MicroRNA-451 regulates p38 MAPK signaling by targeting of Ywhaz and suppresses the mesangial hypertrophy in early diabetic nephropathy. FEBS Lett. 2012;586(1):20–6.
97. Long J, Wang Y, Wang W, Chang BH, Danesh FR. Identification of microRNA-93 as a novel regulator of vascular endothelial growth factor in hyperglycemic conditions. J Biol Chem. 2010;285(30):23457–65.
98. Chen YQ, Wang XX, Yao XM, Zhang DL, Yang XF, Tian SF, et al. MicroRNA-195 promotes apoptosis in mouse podocytes via enhanced caspase activity driven by BCL2 insufficiency. Am J Nephrol. 2011;34(6):549–59.
99. Chen YQ, Wang XX, Yao XM, Zhang DL, Yang XF, Tian SF, et al. Abated microRNA-195 expression protected mesangial cells from apoptosis in early diabetic renal injury in mice. J Nephrol. 2012;25(4):566–76.
100. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. Nature. 2008;454(7200):56–61.
101. Li R, Chung AC, Dong Y, Yang W, Zhong X, Lan HY. The microRNA miR-433 promotes renal fibrosis by amplifying the TGF-beta/Smad3-Azin1 pathway. Kidney Int. 2013;84(6): 1129–44.
102. Zhou Q, Chung AC, Huang XR, Dong Y, Yu X, Lan HY. Identification of novel long noncoding RNAs associated with TGF-beta/Smad3-mediated renal inflammation and fibrosis by RNA sequencing. Am J Pathol. 2014;184(2):409–17.
103. Lan HY, Chung AC. Transforming growth factor-beta and Smads. Contrib Nephrol. 2011; 170:75–82.
104. Kato M, Zhang J, Wang M, Lanting L, Yuan H, Rossi JJ, et al. MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-beta-induced collagen expression via inhibition of E-box repressors. Proc Natl Acad Sci U S A. 2007;104(9):3432–7.
105. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci. 2008;105(30):10513–8.
106. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008;18(10): 997–1006.
107. Volinia S, Visone R, Galasso M, Rossi E, Croce CM. Identification of microRNA activity by targets' reverse eXpression. Bioinformatics. 2010;26(1):91–7.
108. Zampetaki A, Willeit P, Drozdov I, Kiechl S, Mayr M. Profiling of circulating microRNAs: from single biomarkers to re-wired networks. Cardiovasc Res. 2012;93(4):555–62.
109. Argyropoulos C, Wang K, McClarty S, Huang D, Bernardo J, Ellis D, et al. Urinary microRNA profiling in the nephropathy of type 1 diabetes. PLoS One. 2013;8(1):e54662.
110. Barutta F, Tricarico M, Corbelli A, Annaratone L, Pinach S, Grimaldi S, et al. Urinary exosomal microRNAs in incipient diabetic nephropathy. PLoS One. 2013;8(11):e73798.

Chapter 14
microRNA and Kidney Transplantation

Kíra Jelencsics and Rainer Oberbauer

Abstract The kidney serves as the main clearance organ of our body, filtrating and excreting metabolic waste products. Various intrinsic and extrinsic conditions can lead to kidney injury, roughly 0.1 % of the population suffer from end stage renal disease. Renal transplantation reinstitutes an almost normal quality of life; again it is cost effective and thus the preferred treatment of terminal renal failure.

miRNAs play pivotal roles in immune responses and inflammation, which makes them particularly interesting in the field of transplantation and in understanding the molecular pathways of allograft pathologies such as delayed function or cellular and antibody mediated rejection. As kidney biopsy is part of the routine disease monitoring, the identification of miRNA pattern is feasible in different stages of the injury.

Furthermore miRNAs are easy to detect not only in tissue samples but also in body fluids such as blood and urine. Their regulatory capacity of biological processes together with their stability makes them excellent candidates for noninvasive monitoring of kidney pathology. There is an accumulating knowledge about diseases-specific miRNA signatures in distinct kidney injuries. In the following chapter we present the current understanding of miRNAs regulation of intragraft processes after kidney transplantation.

Keywords Kidney • Transplantation • Pathology

Introduction

The kidneys are the main clearance organs of the body. They consist of about one million of functional units called nephrons, which produce an ultrafiltration of the blood and reabsorb necessary substances such as electrolytes and about 99 % of the 180 L filtered water. Main excretory substances are hydrogen ions via ammonium and titrable acidity as well as urea and creatinine. The primary location of the filtration is in the glomerulus at the beginning of the nephron, what is rich in blood

K. Jelencsics • R. Oberbauer (✉)
Medical University of Vienna, Währinger Gürtel 18-20, Vienna 1090, Austria
e-mail: rainer.oberbauer@meduniwien.ac.at

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_14

vessels. The final concentration and the composition of the urine are defined in the renal tubule of the nephron by reabsorption, active and passive secretion.

microRNAs have been implicated as transcriptional regulators in a wide range of biological processes determining cell fate, stress response, proliferation, or death. Immense research efforts have identified many associations between disease mechanisms and specific microRNAs. In kidney cells and tissues there are more than 200 miRNAs identified so far. For example, conditional knockout of Dicer from podocytes leads to glomerular defects and rapid progression to end stage renal disease, suggesting an important role for microRNAs in podocyte function in glomeruli of the kidneys [1].

The kidney disease classification refers to the site of the insult and whether the injury is intrinsic to the kidney or the consequences of a systemic disease such as diabetes, arterial hypertension or exogenous substances such as pathogens, drugs, or X-ray contrast media. If the kidney function falls below 8 %, i.e., equaling a glomerular filtration rate (GFR) of 8 mL/min, renal replacement therapy needs to be initiated. Unambiguously the best option is renal transplantation, which allows for an almost normal life. In 2011, 17,671 kidney transplantations were performed in the USA [2].

Since the transplanted kidney is a precious gift, it is intuitive that it is of utmost importance that the graft half-life needs to be maximized. Currently the half-life for deceased donor organs is roughly 8–10 years whereas for live donor grafts it is around 12–15 years [2, 3]. Among the main risk factors for a reduced long-term patency is the development of immediate post-transplant delayed graft function (DGF). The pathophysiology behind DGF is postischemic acute kidney injury (AKI) which is described in more detail elsewhere [4]. Other important risk factors that impact on long-term graft patency are humoral and cellular rejection, infections and immunosuppressant toxicity.

miRNAs plays a role in development and functional regulation of the immune system, what makes them particularly interesting in the field of organ transplantation. The human genome encodes over 2000 miRNA, but the list is still incomplete, discovery and the validation of the mostly in silico predicted targets are still ongoing. There is an accumulating, yet incomplete knowledge about the crucial role of specific miRNA signatures known to be involved in the recipients' alloimmune responses following organ transplantation (for an overview of the miRNAs involved in dysfunctions related to kidney transplantation, see Table 14.1). A study used a mouse model with targeted Dicer deletion from the renal proximal tubules, where ischemia-induced loss of renal function was attenuated in the Dicer knockout mice. This experiment provided the first evidence of the role of Dicer and associated miRNA production in ischemic AKI [5].

miRNA in Kidney Transplantation

Ischemia-reperfusion injury (IRI)is one of the common causes of AKI, associated with high morbidity and mortality rates. In solid organ transplantation it is an inevitable part of the procedure, and it has very few effective treatment options.

Table 14.1 miRNAs involved in dysfunctions related to kidney transplantation

Name	Regulation	Pathology	Organ	References
let-7i	Upregulated	ABMR	Kidney	[63]
let-7b	Downregulated	AREJ	Kidney	[63]
let7-c	Downregulated	AREJ	Kidney	[60]
miR-10a	Downregulated	AREJ	Urine, kidney	[49, 60]
miR-10b	Downregulated	AREJ	Urine, kidney	[49, 60]
miR-16	Downregulated	AKI	Plasma	[27]
miR-17	Upregulated	DGF	Kidney	[63]
miR-17-3p_MM1	Downregulated	AREJ	Kidney	[70]
miR-18a	Upregulated	DGF	Kidney	[63]
	Upregulated	IRI	Kidney	[33]
miR-20a	Upregulated	DGF	Kidney	[63]
	Upregulated	IRI	Kidney (mouse)	[11]
miR-20	Upregulated	AKI	Kidney	[33]
miR-21	Upregulated	DGF	Kidney	[63]
	Upregulated	ABMR	Kidney	[63]
	Upregulated	IF/TA	Plasma	[71]
	Upregulated	IF/TA	Macrophages, pericyte precursors	[41]
	Upregulated	IRI	Kidney (mouse)	[11]
	Upregulated	AKI	Urine	[28]
miR-21-3p	Upregulated	AKI	Kidney	[21]
miR-23b	Downregulated	AREJ	Kidney	[63]
miR-27b	Downregulated	AREJ	Kidney	[63]
miR-30a-3p	Downregulated	AREJ	Kidney	[60]
miR-30b	Downregulated	AREJ	Kidney	[60]
miR-30b	Downregulated	IF/TA	Kidney	[44]
miR-30c	Downregulated	IF/TA	Kidney	[44]
miR-30c	Downregulated	AREJ	Kidney	[60]
miR-30c-2*	Downregulated	AREJ	Kidney	[63]
miR-30e-3p	Downregulated	AREJ	Kidney	[60]
miR-32	Downregulated	AREJ	Kidney	[60]
miR-99b and miR-99b*	Downregulated	AREJ	Kidney	[63]
miR-106a	Upregulated	DGF	Kidney	[63]
miR-106b	Upregulated	DGF	Kidney	[63]
miR-124	Downregulated	IF/TA	Tubular cells	[72]
miR-125a	Upregulated	AREJ	Kidney	[73]
	Downregulated	AREJ	Kidney	[63]
	Downregulated	ABMR	PBMC, kidney	[67]
miR-125a_MM1	Upregulated	AREJ	Kidney biopsy	[73]
miR-125	Downregulated	AREJ	Kidney	[60]
miR-125b-2*	Downregulated	AREJ	Kidney	[63]

(continued)

Table 14.1 (continued)

Name	Regulation	Pathology	Organ	References
miR-126	Upregulated	IRI	MP	[56]
miR-127	Upregulated	IRI	Kidney (rat)	[20]
miR-132	Upregulated	IRI	Kidney (mouse)	[5]
miR-138	Downregulated	AREJ	Kidney	[63]
miR-139-5p	Downregulated	AREJ	Kidney	[63]
miR-142-3p	Upregulated	AREJ	Kidney	[60]
	Upregulated	tolerance	PBMC	[74]
	Upregulated	CAD	Urine	[64]
miR-142-5p	Upregulated	AREJ	Kidney	[60]
	Upregulated	IF/TA	Kidney	[44]
miR-142	Upregulated	ABMR	PBMC, kidney	[67]
miR-146a	Upregulated	IRI	Kidney (mouse)	[11]
	Upregulated	AREJ	Kidney	[60]
miR-146b	Upregulated	ABMR	Kidney	[63]
	Upregulated	AREJ	Kidney	[60]
miR-150	Upregulated	AREJ	Kidney	[63]
miR-155	Upregulated	AREJ	Kidney	[63]
	Upregulated	IRI	Kidney	[33]
	Upregulated	AREJ	Kidney	[60]
	Upregulated	IF/TA	T-cells	[75]
miR-181	Upregulated	IF/TA	T-cells	[76]
miR-181a	Downregulated	AREJ	Kidney	[63]
miR-181b	Downregulated	AREJ	Kidney	[63]
miR-182	Upregulated	AKI	Kidney	[21]
	Upregulated	CAD	Kidney	[63]
	Upregulated	ABMR	Kidney	[63]
miR-187	Downregulated	IRI	Kidney (mouse)	[11]
miR-192	Downregulated	IRI	Kidney (mouse)	[11]
miR-193b	Downregulated	AREJ	Kidney	[63]
miR_194	Downregulated	ABMR	PBMC, kidney	[67]
	Downregulated	IRI	Kidney (mouse)	[11]
miR-197_MM2	Downregulated	AREJ	Kidney	[73]
miR-199a-3p	Upregulated	IRI	Kidney (mouse)	[11]
miR-200a	Downregulated	AREJ	Kidney	[60]
miR-200c	Upregulated	AKI	Urine	[28]
miR-200 family (miR200-a, b, c, miR-141, miR429)	Upregulated	IF/TA	Mesangial cells	[69]
miR-204	Upregulated	CAD	Urine	[64]
miR-210	Downregulated	AREJ	Urine	[49]
	Downregulated	AKI	Plasma	[49]
miR-211	Downregulated	CAD	Urine	[64]

(continued)

Table 14.1 (continued)

Name	Regulation	Pathology	Organ	References
miR-214	Upregulated	IF/TA	Kidney	[55]
	Upregulated	IRI	Kidney (mouse)	[11]
miR-223	Upregulated	AREJ	PBMC	[60]
miR-296	Upregulated	IRI	MP	[37]
miR-320	Upregulated	AREJ	Kidney	[73]
miR-324-3p	Downregulated	AREJ	Kidney	[73]
miR-326	Downregulated	AREJ	Kidney	[73]
miR-330_MM1	Downregulated	AREJ	Kidney	[73]
miR-342	Upregulated	AREJ	Kidney	[60]
miR-346	Downregulated	AREJ	Kidney	[73]
miR-361-5p	Downregulated	AREJ	Kidney	[63]
miR-362	Upregulated	IRI	Kidney (mouse)	[5]
miR-379	Downregulated	IRI	Kidney (mouse)	[5]
miR-381	Upregulated	AREJ	Kidney	[73]
miR-423	Upregulated	AKI	Urine	[28]
miR-424*	Downregulated	AREJ	Kidney	[63]
miR-455	Downregulated	AREJ	Kidney	[63]
miR-483	Downregulated	AREJ	Kidney	[73]
miR-502-3p	Downregulated	AREJ	Kidney	[63]
miR-506	Upregulated	IF/TA	Kidney	[44]
miR-516-5p	Downregulated	AREJ	Kidney	[73]
miR-524*	Downregulated	AREJ	Kidney	[73]
miR-574-3p	Downregulated	AREJ	Kidney	[63]
miR-602	Upregulated	AREJ	Kidney	[73]
miR-611	Downregulated	AREJ	Kidney	[73]
miR-628	Upregulated	AREJ	Kidney	[73]
miR-629	Upregulated	AREJ	Kidney	[73]
miR-638	Upregulated	AREJ	Kidney	[63]
miR-654	Downregulated	AREJ	Kidney	[73]
miR-658	Upregulated	AREJ	Kidney	[73]
miR-663	Upregulated	ABMR	Kidney	[63]
	Upregulated	AREJ	Kidney	[63]
	Downregulated	AREJ	Kidney	[73]
miR-805	Downregulated	IRI	Kidney (mouse)	[11]
miR-1225-5p		Repair capacity	ARPC	[77]
miR-1228	Upregulated	ABMR	Kidney	[63]
miR-1915		Repair capacity	ARPC	[77]
miR-4640	Downregulated	AKI	Urine	[28]

ABMR antibody-mediated rejection, *AKI* acute kidney injury, *AREJ* acute rejection, *ARPC* adult renal progenitor cell, *CAD* chronic allograft dysfunction, *DGF* delayed graft function, *IRI* ischemia/reperfusion injury, *MP* microparticles (microvesicles and exosomes), *PBMC* peripheral blood mononuclear cells

A hallmark of IRI is necrosis and apoptosis of renal tubular epithelial cells, fibrosis, and proliferation. IRI leads to hypoxia and then during the reperfusion causes oxidative/nitrative stress triggering tissue damage mainly in the renal cortex. The reactive oxygen and nitrogen species induce protein modifications, lipid oxidations, and DNA double strand breaks leading to endothelial dysfunction. Neutrophil adhere and migrate through the endothelium, and inflammatory mediators are released, activating the innate and adaptive immune responses [6]. Although acute complications of I/R injury are reduced by advanced organ protection during surgery and ischemic time and associated organ damage is shorter now, chronic complications such as organ failure are not solved yet. After I/R, the surviving tissue initiates remodeling, in order to maintain adequate organ function. However, the remodeling process might lead eventually into abnormal changes, with ensuing dysfunction and subsequent organ failure. The underlying molecular signaling between cellular components, extracellular matrix, and tissue vascularization during renal remodeling are far from being completely understood.

DGF is the most common complication in the immediate post-transplant period, defined as the necessity for dialysis early after transplantation. This devastating condition has a strong impact of long-term outcomes such as allograft immunogenicity, risk of acute rejection episodes, and long-term survival. However, not all of the molecular processes happening during AKI/DGF are chaotic, in fact some of the events are highly choreographed also by miRNAs [7].

There are different types of miRNA distributions: some miRNAs are widely expressed; others exhibit only limited developmental stage-, tissue-, or cell type-specific patterns. miRNAs can stay local or be released into the body fluids (see examples below), where they remain rather stable. However a special example is miR-21, which was one of the first identified miRNA in human cells [8], appears in most of the kidney dysfunctions. miR-21 is widely expressed normally in many healthy tissues including the kidney but stays inert. In case of injury, miR-21 becomes activated locally at the site of the injury. Most probably there will be more examples identified for such a universal yet specific expression.

It has been shown that post-transplant gene expression profiles of living and deceased donor kidneys are significantly different [9]. Single microRNA may alter the expression of a large number of target genes, thus regulate whole disease-specific pathways and signaling cascades. The identification of the condition-specific miRNA signatures in grafts helps us to reveal the existence of molecular pathways that may be involved in the injury or repair response. For example, redox signaling in cardiac and/or renal ischemia–reperfusion injury may have a critical impact on microRNA biology. For instance, it is likely that I/R results in altered cellular redox signaling events that could potentially lead to altered microRNA levels and hence enhanced proinflammatory or diminished antioxidant gene expression. This will result in the initiation as well as the perpetuation of the cellular injury response (reviewed in [10]).

A set of tissue-specific miRNAs was identified and validated from biopsies taken from mice undergoing IRI (Fig. 14.1): miR-21 and miR-20a expression showed rapid upregulation upon injury, upregulation of miR-146a, miR-199a-3p, and miR-214 occurred after the third day. miR-192 and miR-187 were rapid downregulated,

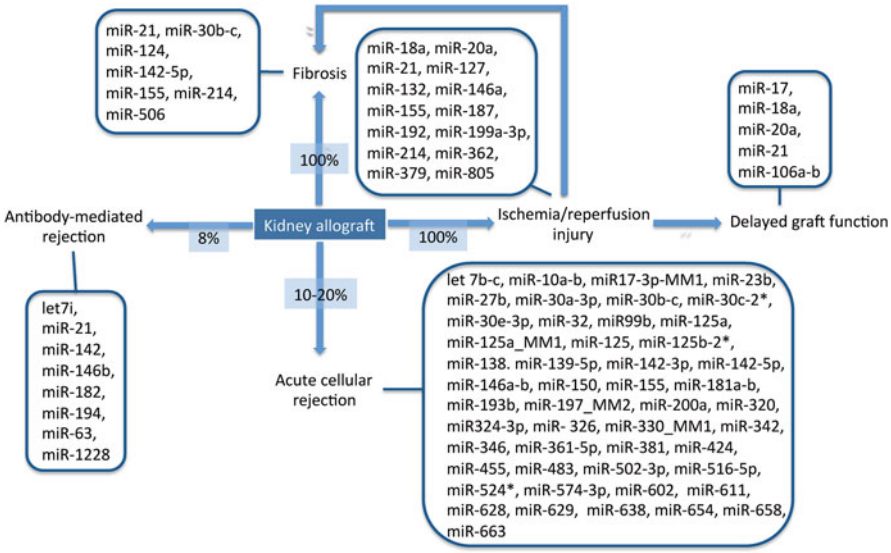

Fig. 14.1 Entity-specific deregulated miRNAs in the transplant. *Percentage*: occurrence of pathological changes

miR-805 and miR-194 downregulated but stayed at similar level to naïve kidney. The expression pattern of these miRNAs after warm ischemia is lymphocyte-independent, suggesting that it is an intrinsic response from the kidney, a very own fingerprint of renal IRI [11].

miR-21 is a particularly interesting miRNA in kidney diseases because it plays a role in several processes that also occur as a result of IRI. We reviewed miR-21 in Box 14.1. miR-20a regulates E2F transcription factors, which are involved in proliferation and apoptosis [12]. This means that miR-21 and miR-20a may prevent apoptosis resulting from IRI and induce proliferation to promote repair [11]. miR-146a is an important modulator of differentiation and function of cells involved in innate and adaptive immunity (reviewed in [13]). The expression of miR-146 is upregulated by inflammatory factors such as interleukin 1 and tumor necrosis factor-α. miR-146 deregulates a number of targets, which are mostly involved in toll-like receptor pathways, bringing a cytokine response as part of the innate immune system and providing a negative feedback loop to TLR-signaling [13]. The delayed miR-146a expression may be required to resolve inflammation and allow for repair following IRI [11]. miR-199 is implicated in a wide variety of cellular and developmental mechanisms. In a cluster together with miR-214 both are involved in skeleton formation and in cancer. miR-199a is involved in various cancer development and progression and cardiomyocytes protection, targeting the Met proto-oncogene [14]. miR-214 improves the metastasis of melanoma. Through the regulation of two activating protein-2 transcription factors, influences a number of genes regulating apoptosis, proliferation, and angiogenesis [15, 16]. miR-192 and

Box 14.1: Review of miR-21

miR-21 is widely expressed in all the tissues and is expressed quite highly in the normal kidney, heart, spleen, liver, and lung as well. However it stays inert, and just upon injury will be the active form upregulated. Thus, inhibition by antisense oligonucleotides acts only at the site of the injury. miR-21 is sequestered most probably in an intracellular compartment, and released into the cytoplasm in response to stress [41]. miR-21 is a highly induced antiapoptotic miRNA during IRI [11], renal and cardiac fibrosis, and contributes to carcinogenesis in prostate and other cancer. miR-21 is highly upregulated after 15 min of renal ischemic preconditioning, and remarkably attenuated IRI induced 4 days later [42] by better renal function, less injury, and fewer apoptotic cells.

miR-21 expression is increased in proliferating tubular epithelial cells, though overexpression of miR-21 alone was not sufficient to prevent TEC death following ischemia (which means rather a fine-tuning of cellular responses to injury). Silencing of miR-21 enhances apoptosis [11], and results in a decrease in expression of collagen 1A1, collagen 1A2, fibronectin, SMA-α, PAI-1 and TGF-β, as well as macrophage infiltration [18]. miR-21−/− mice and wild-type mice treated with anti-miR-21 oligonucleotides suffer far less interstitial fibrosis in response to kidney injury [41].

As several other miRNAs, miR-21 is affected by TGF-β. Renal IRI stimulates TGF-β production, which upregulates miR-21 expression via a cell-extrinsic mechanism. This causes fibrosis, a major complication resulting from renal IRI, where TGF-β plays a central role, increasing total collagen expression and intensifying the matrix accumulation in renal disease [43]. SMAD7, a verified miR-21 target, has been shown to inhibit TGFβ fibrotic effects on TEC by regulating SMAD2 activation [44].

Ischemia or hypoxia upregulates miR-21 in a cell-intrinsic fashion [11]. NADPH oxidase-dependent reactive oxygen species (ROS) regulate the expression and function of miR-21 defining the invasive phenotype of cancer [45]. NADPH oxidase activity is dependent on the ERK MAP kinase activity. miR-21 expression upregulates the ERK-MAPK signaling pathway, which has impacts on global cardiac structure and function through inhibition of sprouty homolog 1 (Spry1) [46]. ERK-MAPK signaling plays a key role in renal IRI as well.

miR-21 also directly targets the tumor suppressor gene-programmed cell death 4 (PDCD4), resulting in cellular proliferation and increased invasiveness of the cells [45]. In murine cardiomyocytes miR-21 was found to protect from hypoxia/reoxygenation-induced cell apoptosis via regulation of its target gene PDCD4 [47].

miR-194 work together with miR-215 in a miRNA network as a regulator of E-cadherin expression. Their expression suppresses' tumor progression, cell migration and invasion in renal cell carcinoma cell line models, whereas knockdown of these miRNAs resulted in enhancing cellular migration and invasion abilities [15]. miR-192 is highly expressed in the kidney [9] and appears to be proportionately upregulated in response to changes observed in TGF-β expression, which explains its progressive decrease following IRI. In cancer development high miR-187 levels are associated with higher survival rates in ovarian cancer patients through targeting the DAB2 protein, which plays role in cell proliferation and tumor progression. miR-187 expression is increased in islets from individuals with type 2 diabetes as well. It negatively influences the glucose-stimulated insulin secretion, potentially involving regulation of HIPK3, which occurs during the pathogenesis of type 2 diabetes [17]. miR-805 expression is decreased significantly in early diabetic nephropathy [18], reflecting on accumulation of activated macrophages in the injured kidney. Godwin et al. observed indeed significant infiltration of macrophages in the kidney by day 14, matching to the rather slow downregulation pattern of miR-805.

Saphiro et al. investigated whether samples that underwent renal injury as a result of IRI can be distinguished from controls based on alterations in miR expression using the above data set described by Godwin et al. They verified that sham and IRI data reflect predictable alterations in miR expression throughout the course of an injury response. Thus miR expression profiling can be used to diagnose IRI and sham controls and miR expression may therefore be useful as a biomarker for IRI [19].

miR-127 was found by another group to protect proximal tubule cells against IRI in rats, being regulated by HIF-1α and targeting KIF3B. Modulation of miR-127 resulted in changes in cell adhesion and cytoskeletal structure [20]. miR-127 is involved in the downregulation of Rtl1, a key gene in placenta formation and in fetal lung development. miR-127 is described as a ubiquitously expressed microRNA, which can be detected in several human and rat tissues apart of kidney and lung cells; for example, it is also expressed in other human epithelial cells such as breast. Since miR-127 is regulated by ischemia it could be a potential tissue damage biomarker as well [20].

Wilflingseder et al. [21] found miR-182-5p and miR-21-3p as a signature of AKI. miR-182-5p was identified as a molecular regulator of post-transplant AKI, strongly correlated with global gene expression changes during AKI. miR-182 is a key regulator in different ischemic models in heart and brain as well [22, 23]. Further, in a natural model of ischemic tolerance, miR-182 was significantly decreased in brains of ground squirrels during hibernation torpor [24]. miR-182 is activated by interleukin-2 and STAT5 and inhibits FOXO1, leading to clonal expansion of T helper cells. Further, BCL2 is a direct target of miR-182-5p as well, suggesting potent antiapoptotic effects. These analogies indicate that miR-182 may play a critical role in the regulation of both ischemic and immunologic injuries [21].

Seven miRNAs were identified in kidneys with DGF, which are all involved in cell death and proliferation: miR-182-5p and miR-106b, miR-20a, miR-21*, miR-18a, miR-17, miR106-a [21]. The overlap with AKI miRNAs may indicate similar activated pathways.

A good example for kidney-related miRNAs found in the body fluids is miR-210. miR-210 is released from renal endothelial cells, among others, in response to hypoxia in AKI. miR-210 is downregulated in urine and has been shown to be present also in the plasma of patients with acute renal allograft rejection compared to control patients with stable transplant function. miR-210 plays role in the molecular response to hypoxia, being induced by the hypoxia-inducible factor in endothelial cells [25]. In mouse model of IRI, miR-210 regulates in kidney tissue renal angiogenesis by activation of the VEGF signaling pathway. It is also involved in other ischemic diseases such as myocardial infarction, in which miR-210 was shown to be upregulated [26]. In patients with AKI plasma levels of miR-210 were independently associated with 28-day mortality but successful antirejection therapy normalized miR-210 levels [27]. It may thus also be used as prognostic biomarker for long-term graft patency.

Ramachandran identified a panel of four miRNAs (miR-21, miR-200c, miR-423, and miR-4640), which are detectable noninvasively in urine of patients undergoing AKI, with 10, 4.5, and 5-fold higher level and 2-fold lower levels, respectively, compared to controls without kidney injury [28]. These miRNAs were able to distinguish between ICU patients with and patients without evidence of AKI ($p < 0.01$).

Members of the miR-200 family are highly enriched in epithelial tissues, in cells, which are able to form metastases. miR-200 promotes the last step of metastasis of cancer cells during their colonization at distant tissues [29]. miR-423 is extensively studied as a biomarker of heart failure [30] but it is also involved in rapidly progressing idiopathic pulmonary fibrosis (together with miR-210) [31], in lupus nephritis, obesity, and Alzheimer disease [28]. In Alzheimer-disease miR-423 is the hub of a network built in the grey and the white matter by co-expressed microRNAs [32].

miR-21 was found upregulated 1.2-fold in blood and urine of patients with clinical diagnosis of AKI by another group [33] as well. Additionally Saikumar et al. found that miR-155 level is 1.5-fold lower in the same samples, compared with healthy volunteers. Similarly to miR-21 miR-155 is detectable in all human tissues, and abundant in some of the organs like kidney, heart, spleen, liver, and lung. It plays a critical role in a wide variety of biological processes, including hematopoiesis [34].

Different cell types release miRNAs by active secretion into microvesicles or exosomes. microRNAs can also be released from damaged cells or through apoptotic bodies, as it happens after myocardial infarction or in atherosclerosis [35]. The microvesicles are containing proteins, cytokines, and miRNAs [36]. Most probably the microvesicles are not just markers, but have intracellular biological roles, e.g., communication or serving as paracrine mediators, as the microvesicles released by endothelial progenitor cells (EPC) during AKI [37]. EPCs are circulating, bone marrow-derived precursors, which are recruited at tissue injury, like in kidney allograft after IRI, and promoting regeneration by secreting pro-angiogenic factors. Microvesicles released from these progenitor cells activate an angiogenic program in endothelial cells by horizontal miRNA transfer, resulting in protection from AKI by enhanced tubular cell proliferation, reduced apoptosis, and leukocyte infiltration. The protective effect results in decreases in serum creatinine/BUN levels and improved histological signs of microvascular and tubular injury.

Microvesicles protect against chronic kidney damage as well by inhibiting capillary rarefaction, glomerulosclerosis, and tubulointerstitial fibrosis. The renoprotective effect can be inhibited by treatment with RNase, Dicer knock-down in the progenitor cells, or depletion of miR-126 and miR-296 by antisense oligonucleotides (reviewed in [38]). The pro-angiogenic and anti-apoptotic miR-126 and miR-296 have a key role in the renoprotective effect of EPC-derived microvesicles among several other miRNAs. The miRNAs target cell proliferation, angiogenesis, and inhibition of apoptosis as well [37].

Isolated exosomes from T-cells modulate the immune response, which makes exosomes a potential immunotherapeutic candidate in transplantation setting [39]. After AKI there is a bidirectional exchange of genetic information between mesenchymal stem cells (MSC) and the surviving tubular cells, driven by microvesicles containing certain miRNA and mRNA. This might induce the injured cells to start dedifferentiation and tissue-self repair [40] instead of remodeling into abnormal changes with subsequent organ failure.

The most immediate clinical benefit of miRNAs is the opportunity to use them as biomarkers for diagnosis, prognosis, and response to therapy in renal allografts (Fig. 14.2). The current diagnostic algorithm of renal allograft deterioration almost always includes an invasive biopsy at the end. However, longitudinal and frequent

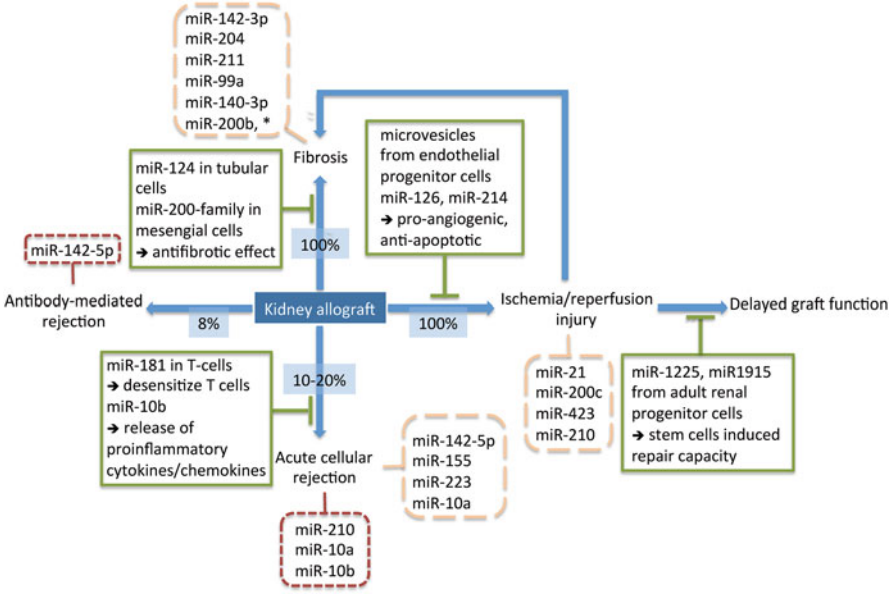

Fig. 14.2 Putative noninvasive biomarkers and protective intervention possibilities in dysfunctions related to kidney transplantation. *Percentage*: occurrence of pathological changes. *Dotted line*: biomarkers found in blood, *dashed line*: biomarkers found in urine

biopsies have their natural limits. miRNAs are detectable not just in tissue samples, but in blood, urine, and other body fluids as well, occurring with different expression profiles predicting allograft status. miRNAs are present before morphological detectable changes occurred or non-reversible injury happened. For example, miR-21, which plays a role in most of kidney injuries, is produced already at renal ischemic preconditioning (short, non-lethal episodes), which is known to reduce injury caused by subsequent IRI [42]. Implementation of such noninvasive molecular biomarkers will lower the costs and risks of DGF, increase the sensitivity of the test, and improve serial monitoring of kidney injury. Recently the identification and in situ detection of miRNAs even from small amounts are feasible, enabling analysis of small biopsies, limited volumes of body fluids, or even formalin-fixed paraffin-embedded archival material [48].

Next to the noninvasive accessibility another advantage of miRNAs is their stable form in urine and other secreted fluids, in the exosomes or bound to the assembly protein Argonaut. The expression of a highly abundant miRNA (miR-21) was shown to be unaffected by incubation of urine samples at room temperature for up to 24 h as well as a number of freeze/thaw cycles [49]. The Argonaute proteins bind miRNAs' guide strand after its cleavage by Dicer. Together they form the core RNA induced silencing complex (RISC), Argonaute proteins will cleave the target mRNA strand. Furthermore miRNAs are 10 times more stable than mRNAs, what makes them better candidates as biomarkers. Accurate data can be measured even from degraded total RNA samples, when most of the mRNAs are already lost [50].

However, next to the identification of putative miRNA biomarkers it is important to determine whether miR expression has prognostic value and whether miR expression returns to baseline following a healing response [19]. It has to be clearly stated that so far no validated miRNA biomarker for kidney injury has been approved for routine care. Another problem is that there is yet no stable endogenous miRNA for normalization identified. Thus the measurements rely on indirect mechanisms of normalization, such as adding unrelated synthetic miRNA to blood samples during RNA isolation (for example, in [49] cel-miR-39 was used). Furthermore globin mRNA in red blood cells may influence expression profiling of whole blood specimens. This can be excluded by using isolated leukocytes globin reduction, and mathematical depletion to lighten the confounding globin molecular signatures [51]. Furthermore, analysis of circulating miRNAs of patients with acute illnesses such as myocardial infarction or AKI - though all the benefits - is costly and time-consuming, because of the RNA extraction, the reverse transcription and the subsequent quantitative real-time PCR analysis. Thus, even if a good candidate miRNA is identified, the difficulty lies in the translation of the diagnostic procedure into future clinical use. A bench-to-bedside test is an unmet need.

Though a relative high number of kidney transplantation is performed yearly, the number of candidates on the wait lists increases yearly with around 2–4 % according to the data from OPTN/SRTR [3]. The gap between successful transplantation and wait list could be smaller by using more marginal donor kidneys and DCD

kidneys (donation after cardiac death). There is an unmet need to improve the functions of these suboptimal organs or treat injured grafts in post-transplant complications in order to achieve longer graft half-life and minimalize the possibility of DGF. The search for contributing miRNAs in the context of renal injury reveals the existence of molecular pathways that may be involved in the injury or repair response that can be a putative target for new therapy options in order to prevent damage or promote healing.

miRNA activity can be inhibited by several antisense strategies, such as chemically modified antisense oligonucleotide inhibitors (antagomirs) or the transgenic introduction of tandem miRNA-binding site repeats (known as Decoy or Sponge technologies). Lorenzen et al. showed recently that miR-24 antagonism prevents renal reperfusion injury in vivo. The treatment with an antisense oligonucleotide targeting miR-24 before the induction of ischemic reperfusion injury resulted in a significantly improved kidney function [52]. In other cases, in diseases in which miRNAs are downregulated, restoring miRNA function might be desirable, and can be achieved by administration of double-stranded miRNA mimics. miR-10b is significantly downregulated in rejected allografts. Transfecting miR-10b into human renal glomerular endothelial cells diminished key features of acute allograft rejection such as release of pro-inflammatory cytokines/chemokines whereas transfection of miR-10b inhibitor had opposite effects [53]. The addition of oligonucleotides by intravenous injection [54] or by different delivery systems (viral or non-viral) is feasible as well. However toxicity derived from off-target effects and from activation of the innate and adaptive immune response and instability of the oligonucleotides (unmodified oligonucleotide is eliminated within minutes) are still issues. From this point of view the kidney is a special target for miRNA therapy, since after systemic administration, the inhibitor oligonucleotides accumulate there abundantly. However it is of note that inhibition therapies should act local to avoid any potential disadvantages of modulating pathways in other tissues and organs such as the heart [55].

Vascular regeneration is a viable therapeutic option after IRI, and the modulation of vascular miRNAs might be a good choice for treating disease activity. Overexpression miR-126 derived from the hematopoietic compartment protects against renal IRI, preserving capillary density and increasing bone marrow-derived peritubular capillary epithelial cells. miR-126 selectively enhances the mobilization of vasculogenic stem and progenitor cells. Protection of the kidney and vascular integrity correlated significantly with the levels of circulating vascular progenitor cells [56]. Bijkerk et al. hypothesize that miR-126 could be increased in the tubular epithelial cells by lateral transfer through the fusion of miR-126 rich, blood cell derived microvesicles as it was mentioned in the case of the endothelial progenitor cell-derived miR-126-rich microparticles [37]. An alternative way could be the microparticles production of the bone marrow-derived epithelial cells, since such particles are able to cross the tubular basement membrane as well [57]. Moreover, platelets are also excellent miR-126 sources, thus can serve as transporters to the site of the injury [58]. In order to improve miR-driven therapies, revealing of miRNA mode of actions is inevitable.

Additional Examples of miRNAs Involved in Regulation of Post-transplant Events

Acute cellular rejection: Acute rejection of grafts both from living and deceased donors occurs at an incidenceof roughly 10 % at most transplant centers [59]. It is still a common risk after kidney transplantation, and currently biopsy is the only way of diagnosis. The full understanding of the molecular processes of rejection and the search for noninvasive predictive and diagnostic biological markers of acute cellular and humoral allograft rejection remain elusive. A number of miRNAs were already found in urine and plasma, associated with acute cellular rejection and proposed as biomarker, and miRNAs from biopsy samples are found to be involved in acute rejection as well. Anglicheau et al. found among 53 significantly differentially regulated miRNAs in acute rejection the intragraft levels of miR-142-5p, -155, -223, -10b, -30a-3p, and let-7c to be diagnostic for acute rejection [60], and most of these miRNAs are involved in IRI or chronic allograft dysfunctions (CADs) too. Additionally miR-142-5p, -155, and miR-223 are detected in PBMCs of renal transplant recipient, with high specificity and sensitivity of miR-223 to diagnose acute rejection [61], which makes it a potential candidate of noninvasive diagnostics pend though validation is still needed.

miR-142 was found to be involved in chronic antibody-mediated rejection and fibrosis as well, associated with immunological disorders rather than renal dysfunction. miR-142 is part of the multiplex sclerosis miRNA signature in PBMCs and plays role in mammalian hematopoiesis and in lupus erythematous as well, produced by leukocytes [62]. miR-155, a typical multifunctional miRNA, is widely expressed in the body, and found upregulated in AKI as well. It is involved in various pathological pathways in haematopoietic lineage differentiation, immunity, inflammation, cancer, and cardiovascular diseases. It is a key player in adaptive immunity and T-cell-mediated antibody response in regulating Th1/Th2 cell differentiation, and T-cell receptor stimulation is known to induce miR-155 expression [34]. miR-10b upregulation in human renal glomerular endothelial cells weakened key features of allograft rejection such as release of pro-inflammatory cytokines/chemokines, whereas inhibition of miR-10b had opposite effects [52].

Wilflingseder et al. identified a set of miRNAs in kidney biopsies, which were able to distinguish between acute rejection, antibody-mediated rejection, and DGF [63]. In AREJ, four miRNAs were found to be significantly upregulated (miR-150, miR-155, miR-663, and miR-638). miR-150 plays an important role in B-cell development and controls c-Myb expression in vivo. Overexpression of miR-663 initiates an inflammatory response of endothelial cells. Both miR-638 and miR-663 were found to be upregulated in PBMCs and is involved in lupus nephritis, indicating a high inflammatory state in kidney tissue [63]. miR-663, miR-30c, miR-139, and let-7b were also associated with CAD [64].

As it was mentioned already, miR-210 is found to be strongly downregulated in ischemic diseases, such as AKI, but in urine sample of patients with undergoing acute rejection as well [49]. Next to miR-210, 10b is downregulated not just in rejected allografts but in urine too, whereas 10a is upregulated after acute rejection.

miR-10a regulates the proinflammatory phenotype in athero-susceptible endothelium [65] and thus influences the invasion of immune cells during acute cellular rejection. But among these three urinal miRNAs miR-210 is the only one, which can differ between acute rejection and urinary tract infection of allograft, furthermore it can predict GFR level in 1 year after transplantation [49]. In serum only a limited number, two miRNAs were found from the set already identified in graft tissue. Compared to 1 year post-transplant samples miR-223 and miR-10a showed upregulation [66].

Antibody-mediated rejection: Alloantibodies to antigens expressed by endothelium of allograft may cause sequential types of antibody-mediated rejection: from the accidentally hyperacute rejection, the acute and the chronic rejection, and even apparent graft acceptance (accommodation). Chronic antibody-mediated rejection is a major risk factor for long-term graft survival in kidney transplantation. miR-142 [67] from both tissue biopsies and peripheral blood PBMCs was found to influence the expression of numerous genes involved in chronic antibody-mediated rejection. miR-142-5p might be potential biomarker allowing a very good discrimination of the patients with chronic antibody-mediated rejection ($p=0.0056$). It is carried to the kidney by leukocytes against local inflammatory process, underlining its possibility to use as target for therapeutic settings.

miR-663, miR-146b-5p, miR-1228, let-7i, miR-21*, and miR-182 were found specific to antibody-mediated rejection [63]. As it was already mentioned, miR-182 plays a significant role in AKI and DGF as well, being upregulated in IRI [21]. miR-663 and miR-146b were also upregulated in biopsies developing acute rejection ([63] and [60], respectively). The accordance of these miRNAs indicates that processes involved in DGF and acute rejection are also important in the pathogenesis of the antibody-mediated rejection.

Chronic allograft dysfunction: Tissue fibrosis refers to the accumulation of collagen-richextracellular matrix by fibroblasts as a response to tissue injury, causing tubular atrophy with decrease in tubular diameter and loss of function. AKI is a contributory factor in the development of CAD, although this depends on severity, duration, frequency, and context of the underlying disease. CAD is a common complication after kidney transplantation, also mentioned as chronic kidney dysfunction (CKD). TGF-β is a key regulator of fibrosis, regulating miRNAs among many other molecules involved in fibrosis. In turn, miRNAs regulate signaling components of TGF-β. Inhibitors of TGF-β are protective against the development of renal tubulointerstitial fibrosis [64].

Inhibition of miR-21 ameliorates injury and fibrosis after IRI [41]. miR-21, which appears in most of the kidney diseases as an early response to stress, is amplifying injury and fibrosis via inhibition of lipid metabolism and increased oxygen radical production (see Box 14.1). Next to miR-21, miR-142-3p, 5p and the cluster comprising miR-506 showed significant, 2–7-times upregulation in interstitial fibrotic biopsies whereas miRNA miR-30b and 30c were downregulated [44]. Scian et al. found that miR-142-3p together with miR-204, miR-107, miR-211, and miR-32 were differently expressed in allograft of patients with CAD. Furthermore, differential expression of miR-142-3p, miR-204, and miR-211 was also observed between patient groups in urine samples [64].

Maluf et al. tested the miRNA profile in urine cell pellets of 125 kidney transplant recipients diagnosed with CAD with interstitial fibrosis and tubular atrophy and those recipients with normal graft function. In a longitudinal evaluation a subset (miR-99a, miR-140-3p, miR-200b, and miR-200*) of these miRNAs was found to be differentially expressed before histological allograft injury was evident [68]. The differences in the results might be due to different experimental techniques, assay platforms, or different characteristics of the patients, what makes findings hard to compare.

miR-21 and miR-214 are both induced by TGF-β stimulation. miR-214 can be found in cells of the damaged glomerulus and tubules as well as in infiltrating immune cells in diseased tissue. Anti-miR-214 treatment before unilateral ureteral obstruction (a model for tubulointerstitial fibrosis) attenuated 93 % of the following fibrotic tissue damage. Thus miR-214 may represent a novel antifibrotic treatment in the kidney [55]. miR-200b (part of the miR200 family) inhibits the epithelial-mesenchymal transition by which renal fibroblasts are generated, via TGF-β stimulation [69]. miR-200c was already found in urine samples of patients with AKI and miR-200a in biopsies, which underwent acute renal rejection.

Conclusion

Kidney transplantation is the preferred therapy for patients with end stage renal failure. Though it is relatively easy to accomplish, post-transplant complications, like ischemia/reperfusion injury, acute cellular rejection, or fibrosis, are still an issue. The improvement of graft-functions are especially important for kidneys from deceased donors, in what the possibility of a dysfunction is even higher, though the need on these organs is increasing, due to the continuously growing wait list for kidneys. miRNAs are essential for renal development, homoeostasis, and cell phenotype and are involved in the pathology of renal diseases. Recent studies in renal allograft recipients showed that miRNAs are highly deregulated in post-transplant episodes, and regulate particular functions, such as intercellular communication, inflammation, injury, rejection, or repair capacity. The miRNA research revealed many unknown molecular networks related to the pathology of kidney transplantation, allowing to find better biomarkers for disease monitoring and more accurate intervention points in order to save allograft and improve its long-term functions. The opportunity of inhibiting or mimicking of miRNA with biotechnologically engineered oligonucleotides offers new and effective therapeutic options. However, many roles that individual miRNA has in the regulation of the immune system are still being discovered, and many targets of specific miRNAs remain yet unclear. Bioinformatics analyses have predicted thousands of miRNA-target pairs but only a small proportion of these have been validated. Furthermore most of the miRNAs are universal molecules, being involved in many organs, so many safety concerns must be still resolved, from the avoidance of toxicity derived off-target effects to activation of the innate and adaptive immune response.

Since miRNAs are highly pleiotropic and act differently depending on the cell or tissue type, a single miRNA is unlikely to useful as biomarker for diagnose. Instead,

a network of correlated miRNAs must be considered. After the identification of these miRNA sets clinical utility needs to be determined in a larger prospective study. Furthermore, though the so far identified miRNAs are promising, work is still needed to translate these findings into clinical routine, finding also cost-effective technologies to use on a daily bases. Despite of the remaining open questions, we believe that the day when miRNAs will play important role of the diagnostic and therapeutic routines, facilitating and improving many lives is approaching (Table 14.1).

References

1. Harvey SJ, Jarad G, Cunningham J, Goldberg S, Schermer B, Harfe BD, et al. Podocyte-specific deletion of dicer alters cytoskeletal dynamics and causes glomerular disease. J Am Soc Nephrol. 2008;19(11):2150–8.
2. Collins AJ, Foley RN, Chavers B, Gilbertson D, Herzog C, Ishani A, et al. US renal data system 2013 annual data report. Am J Kidney Dis. 2014;63(1 Suppl):A7.
3. Matas AJ, Smith JM, Skeans MA, Thompson B, Gustafson SK, Stewart DE, et al. OPTN/SRTR 2013 annual data report: kidney. Am J Transplant. 2015;15(S2):1–34.
4. Kainz A, Wilflingseder J, Mitterbauer C, Haller M, Burghuber C, Perco P, et al. Steroid pretreatment of organ donors to prevent postischemic renal allograft failure: a randomized, controlled trial. Ann Intern Med. 2010;153(4):222–30.
5. Wei Q, Bhatt K, He HZ, Mi QS, Haase VH, Dong Z. Targeted deletion of Dicer from proximal tubules protects against renal ischemia-reperfusion injury. J Am Soc Nephrol. 2010;21(5):756–61.
6. Weight SC, Bell PR, Nicholson ML. Renal ischaemia–reperfusion injury. Br J Surg. 1996;83(2):162–70.
7. Bhatt K, Mi QS, Dong Z. microRNAs in kidneys: biogenesis, regulation, and pathophysiological roles. Am J Physiol Renal Physiol. 2011;300(3):F602–10.
8. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A. 2004;101(9):2999–3004.
9. Mueller TF, Reeve J, Jhangri GS, Mengel M, Jacaj Z, Cairo L, et al. The transcriptome of the implant biopsy identifies donor kidneys at increased risk of delayed graft function. Am J Transplant. 2008;8(1):78–85.
10. Marin T, Gongol B, Chen Z, Woo B, Subramaniam S, Chien S, et al. Mechanosensitive microRNAs-role in endothelial responses to shear stress and redox state. Free Radic Biol Med. 2013;64:61–8.
11. Godwin JG, Geb X, Stephana K, Jurischb A, Tullius SG, Iacominia J. Identification of a microRNA signature of renal ischemia reperfusion injury. Proc Natl Acad Sci U S A. 2010;107(32):14339–44.
12. Sylvestre Y, De Guire V, Querido E, Mukhopadhyay UK, Bourdeau V, Major F, et al. An E2F/miR-20a autoregulatory feedback loop. J Biol Chem. 2007;282(4):2135–43.
13. Rusca N, Monticelli S. MiR-146a in immunity and disease. Mol Biol Int. 2011;2011:437301.
14. Gu S, Chan WY. Flexible and versatile as a chameleon-sophisticated functions of microRNA-199a. Int J Mol Sci. 2012;13(7):8449–66.
15. Penna E1, Orso F, Cimino D, Tenaglia E, Lembo A, Quaglino E, et al. microRNA-214 contributes to melanoma tumour progression through suppression of TFAP2C. EMBO J. 2011;30(10):1990–2007.
16. Sun Y, Koo S, White N, Peralta E, Esau C, Dean NM, et al. Development of a micro-array to detect human and mouse microRNAs and characterization of expression in human organs. Nucleic Acids Res. 2004;32(22), e188.

17. Locke JM, da Silva XG, Dawe HR, Rutter GA, Harries LW. Increased expression of miR-187 in human islets from individuals with type 2 diabetes is associated with reduced glucose-stimulated insulin secretion. Diabetologia. 2014;57(1):122–8.
18. Chen YQ, Wang XX, Yao XM, Zhang DL, Yang XF, Tian SF, et al. Abated microRNA-195 expression protected mesangial cells from apoptosis in early diabetic renal injury in mice. J Nephrol. 2012;25(4):566–76.
19. Shapiro MD, Bagley J, Latz J, Godwin JG, Ge X, Tullius SG, et al. MicroRNA expression data reveals a signature of kidney damage following ischemia reperfusion injury. PLoS One. 2011;6(8):e23011.
20. Aguado-Fraile E, Ramos E, Sáenz-Morales D, Conde E, Blanco-Sánchez I, Stamatakis K, et al. miR-127 protects proximal tubule cells against ischemia/reperfusion: identification of Kinesin family member 3B as miR-127 target. PloS One. 2012;7(9):e44305.
21. Wilflingseder J, Sunzenauer J, Toronyi E, Heinzel A, Kainz A, Mayer B, et al. Molecular pathogenesis of post-transplant acute kidney injury: assessment of whole-genome mRNA and miRNA profiles. PLoS One. 2014;9(8):e104164.
22. Wei L, Wang M, Qu X, Mah A, Xiong X, Harris AG, et al. Differential expression of microRNAs during allograft rejection. Am J Transplant. 2012;12(5):1113–23.
23. Lee ST, Chu K, Jung KH, Yoon HJ, Jeon D, Kang KM, et al. MicroRNAs induced during ischemic preconditioning. Stroke. 2010;41(8):1646–51.
24. Lee YJ, Johnson KR, Hallenbeck JM. Global protein conjugation by ubiquitin-like-modifiers during ischemic stress is regulated by microRNAs and confers robust tolerance to ischemia. PLoS One. 2012;7(10), e47787.
25. Fasanaro P, D'Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, et al. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. J Biol Chem. 2008;283(23):15878–83.
26. D'Alessandra Y, Devanna P, Limana F, Straino S, Di Carlo A, Brambilla PG, et al. Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. Eur Heart J. 2010;31(22):2765–73.
27. Lorenzen JM, Kielstein JT, Hafer C, Gupta SK, Kumpers P, Faulhaber-Walter R, et al. Circulating miR-210 predicts survival in critically ill patients with acute kidney injury. Clin J Am Soc Nephrol. 2011;6(7):1540–6.
28. Ramachandran K, Saikumar J, Bijol V, Koyner JL, Qian J, Betensky RA, et al. Human miRNome profiling identifies microRNAs differentially present in the urine after kidney injury. Clin Chem. 2013;59(12):1742–52.
29. Dykxhoorn DM. MicroRNAs and metastasis: little RNAs go a long way. Cancer Res. 2010;70(16):6401–6.
30. Goren Y, Kushnir M, Zafrir B, Tabak S, Lewis BS, Amir O. Serum levels of microRNAs in patients with heart failure. Eur J Heart Fail. 2012;14(2):147–54.
31. Oak SR, Murray L, Herath A, Sleeman M, Anderson I, Joshi AD, et al. A micro RNA processing defect in rapidly progressing idiopathic pulmonary fibrosis. PLoS One. 2011;6(6), e21253.
32. Bhattacharyya M, Bandyopadhyay S. Studying the differential co-expression of microRNAs reveals significant role of white matter in early Alzheimer's progression. Mol Biosyst. 2013;9(3):457–66.
33. Saikumar J, Hoffmann D, Kim TM, Gonzalez VR, Zhang Q, Goering PL, et al. Expression, circulation, and excretion profile of microRNA-21, -155, and -18a following acute kidney injury. Toxicol Sci. 2012;129(2):256–67.
34. Faraoni I, Antonetti FR, Cardone J, Bonmassar E. miR-155 gene: a typical multifunctional microRNA. Biochim Biophys Acta. 2009;1792(6):497–505.
35. Zernecke A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, Denecke B, et al. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci Signal. 2009;2(100):ra81.
36. Agarwal A, Fanelli G, Letizia M, Tung SL, Boardman D, Lechler R, et al. Regulatory T cell-derived exosomes: possible therapeutic and diagnostic tools in transplantation. Front Immunol. 2014;5:555.

37. Cantaluppi V, Gatti S, Medica D, Figliolini F, Bruno S, Deregibus MC, et al. Microvesicles derived from endothelial progenitor cells protect the kidney from ischemia–reperfusion injury by microRNA-dependent reprogramming of resident renal cells. Kidney Int. 2012;82:412–27.
38. Lorenzen J, Batkai S, Thum T. Regulation of cardiac and renal ischemia–reperfusion injury by microRNAs. Free Radic Biol Med. 2013;64:78–84.
39. Yu X, Huang C, Song B, Xiao Y, Fang M, Feng J, et al. CD4+ CD25+ regulatory T cells-derived exosomes prolonged kidney allograft survival in a rat model. Cell Immunol. 2013;285(1–2):62–8.
40. Humphreys BD, Bonventre JV. Mesenchymal stem cells in acute kidney injury. Annu Rev Med. 2008;59:311–25.
41. Chau BN, Xin C, Hartner J, Ren S, Castano AP, Linn G, et al. MicroRNA-21 promotes fibrosis of the kidney by silencing metabolic pathways. Sci Transl Med. 2012;4(121):121ra18.
42. Xu X, Kriegel AJ, Liu Y, Usa K, Mladinov D, Liu H, Fang Y, et al. Delayed ischemic preconditioning contributes to renal protection by upregulation of miR-21. Kidney Int. 2012;82(11):1167–75.
43. Creely JJ, DiMari SJ, Howe AM, Haralson MA. Effects of transforming growth factor-beta on collagen synthesis by normal rat kidney epithelial cells. Am J Pathol. 1992;140(1):45–55.
44. Ben-Dov IZ, Muthukumar T, Morozov P, Mueller FB, Tuschl T, Suthanthiran M. MicroRNA sequence profiles of human kidney allografts with or without tubulointerstitial fibrosis. Transplantation. 2012;94(11):1086–94.
45. Jajoo S, Mukherjea D, Kaur T, Sheehan KE, Sheth S, Borse V, Rybak LP, et al. Essential role of NADPH oxidase-dependent reactive oxygen species generation in regulating microRNA-21 expression and function in prostate cancer. Antioxid Redox Signal. 2013;19(16):1863–76.
46. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature. 2008;456(7224):980–4.
47. Cheng Y, Zhu P, Yang J, Liu X, Dong S, Wang X, Chun B, et al. Ischaemic preconditioning-regulated miR-21 protects heart against ischaemia/reperfusion injury via anti-apoptosis through its target PDCD4. Cardiovasc Res. 2010;87(3):431–9.
48. O'Connell RM, Zhao JL, Rao DS. MicroRNA function in myeloid biology. Blood. 2011;118(11):2960–9.
49. Lorenzen JM, Volkmann I, Fiedler J, Schmidt M, Scheffner I, Haller H, et al. Urinary miR-210 as a mediator of acute T-cell mediated rejection in renal allograft recipients. Am J Transplant. 2011;11(10):2221–7.
50. Jung M, Schaefer A, Steiner I, Kempkensteffen C, Stephan C, Erbersdobler A, et al. Robust microRNA stability in degraded RNA preparations from human tissue and cell samples. Clin Chem. 2010;56(6):998–1006.
51. Hartono C, Muthukumar T, Suthanthiran M. Noninvasive diagnosis of acute rejection of renal allografts. Curr Opin Organ Transplant. 2010;15(1):35–41.
52. Lorenzen JM, Kaucsar T, Schauerte C, Schmitt R, Rong S, Hubner A, et al. MicroRNA-24 antagonism prevents renal ischemia reperfusion injury. J Am Soc Nephrol. 2014;25(12): 2717–29.
53. Liu X, Dong C, Jiang Z, Wu WK, Chan MT, Zhang J, et al. MicroRNA-10b downregulation mediates acute rejection of renal allografts by derepressing BCL2L11. Exp Cell Res. 2015;333(1):155–63.
54. Elmen J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S, et al. LNA-mediated microRNA silencing in non-human primates. Nature. 2008;452(7189):896–9.
55. Denby L, Ramdas V, Lu R, Conway BR, Grant JS, Dickinson B, et al. MicroRNA-214 antagonism protects against renal fibrosis. J Am Soc Nephrol. 2014;25(1):65–80.
56. Bijkerk R, van Solingen C, de Boer HC, van der Pol PKM, de Bruin RG, van Oeveren-Rietdijk AM, Lievers E, Schlagwein N, van Gijlswijk DJ, Roeten MK, Neshati Z, de Vries AA, Rodijk M, Pike-Overzet K, van den Berg YW, van der Veer EP, Versteeg H, Reinders ME, et al. Hematopoietic microRNA-126 protects against renal ischemia/reperfusion injury by promoting vascular integrity. J Am Soc Nephrol. 2014;25:8.

57. Borges FT, Melo SA, Ozdemir BC, Kato N, Revuelta I, Miller CA, et al. TGF-beta1-containing exosomes from injured epithelial cells activate fibroblasts to initiate tissue regenerative responses and fibrosis. J Am Soc Nephrol. 2013;24(3):385–92.
58. Nagalla S, Shaw C, Kong X, Kondkar AA, Edelstein LC, Ma L, et al. Platelet microRNA-mRNA coexpression profiles correlate with platelet reactivity. Blood. 2011;117(19):5189–97.
59. USRD USRDS. 2014 Annual data report: epidemiology of kidney disease in the United States. 2014.
60. Anglicheau D, Sharma VK, Ding R, Hummel A, Snopkowski C, Dadhania D, et al. MicroRNA expression profiles predictive of human renal allograft status. Proc Natl Acad Sci U S A. 2009;106(13):5330–5.
61. Liu XY, Xu J. The role of miR-223 in the acute rejection after kidney transplantation. Chin J Cell Mol Immunol. 2011;27(10):1121–3.
62. Ding S, Liang Y, Zhao M, Liang G, Long H, Zhao S, et al. Decreased microRNA-142-3p/5p expression causes CD4+ T cell activation and B cell hyperstimulation in systemic lupus erythematosus. Arthritis Rheum. 2012;64(9):2953–63.
63. Wilflingseder J, Regele H, Perco P, Kainz A, Soleiman A, Muhlbacher F, et al. miRNA profiling discriminates types of rejection and injury in human renal allografts. Transplantation. 2013;95(6):835–41.
64. Scian MJ, Maluf DG, David KG, Archer KJ, Suh JL, Wolen AR, et al. MicroRNA profiles in allograft tissues and paired urines associate with chronic allograft dysfunction with IF/TA. Am J Transplant. 2011;11(10):2110–22.
65. Fang Y, Shi C, Manduchi E, Civelek M, Davies PF. MicroRNA-10a regulation of proinflammatory phenotype in athero-susceptible endothelium in vivo and in vitro. Proc Natl Acad Sci USA. 2010;107(30):13450–5.
66. Betts G, Shankar S, Sherston S, Friend P, Wood KJ. Examination of serum miRNA levels in kidney transplant recipients with acute rejection. Transplantation. 2014;97(4):e28–30.
67. Danger R, Paul C, Giral M, Lavault A, Foucher Y, Degauque N, et al. Expression of miR-142-5p in peripheral blood mononuclear cells from renal transplant patients with chronic antibody-mediated rejection. PLoS One. 2013;8(4):e60702.
68. Maluf DG, Dumur CI, Suh JL, Scian MJ, King AL, Cathro H, et al. The urine microRNA profile may help monitor post-transplant renal graft function. Kidney Int. 2014;85(2):439–49.
69. Oba S, Kumano S, Suzuki E, Nishimatsu H, Takahashi M, Takamori H, et al. miR-200b precursor can ameliorate renal tubulointerstitial fibrosis. PloS One. 2010;5(10):e13614.
70. Sui W, Lin H, Peng W, Huang Y, Chen J, Zhang Y, et al. Molecular dysfunctions in acute rejection after renal transplantation revealed by integrated analysis of transcription factor, microRNA and long noncoding RNA. Genomics. 2013;102(4):310–22.
71. Glowacki F, Savary G, Gnemmi V, Buob D, Van der Hauwaert C, Lo-Guidice JM, et al. Increased circulating miR-21 levels are associated with kidney fibrosis. PLoS One. 2013;8(2):e58014.
72. Zell S, Schmitt R, Witting S, Kreipe HH, Hussein K, Becker JU. Hypoxia induces mesenchymal gene expression in renal tubular epithelial cells: an in vitro model of kidney transplant fibrosis. Nephron Extra. 2013;3(1):50–8.
73. Sui W, Dai Y, Huang Y, Lan H, Yan Q, Huang H. Microarray analysis of microRNA expression in acute rejection after renal transplantation. Transpl Immunol. 2008;19(1):81–5.
74. Danger R, Pallier A, Giral M, Martinez-Llordella M, Lozano JJ, Degauque N, et al. Upregulation of miR-142-3p in peripheral blood mononuclear cells of operationally tolerant patients with a renal transplant. J Am Soc Nephrol. 2012;23(4):597–606.
75. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, et al. Requirement of bic/microRNA-155 for normal immune function. Science. 2007;316(5824):608–11.
76. Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, et al. miR-181a is an intrinsic modulator of T cell sensitivity and selection. Cell. 2007;129(1):147–61.
77. Sallustio F, Serino G, Costantino V, Curci C, Cox SN, De Palma G, et al. miR-1915 and miR-1225-5p regulate the expression of CD133, PAX2 and TLR2 in adult renal progenitor cells. PloS One. 2013;8(7):e68296.

Chapter 15
microRNA and Wound Healing

Jaideep Banerjee and Chandan K. Sen

Abstract microRNAs (miRNAs) are small noncoding RNA molecules which play pivotal roles in wound healing. The increased expression of certain genes and expression of some others represent a key component of the wound biology and are largely under the regulation of naturally occurring miRNAs. Understanding the dysregulated miRNAs in chronic wound biology will therefore enable the development of newer therapies. This chapter focuses on the miRNAs that can be potentially targeted for improving skin wound healing and the challenges in miRNA therapy, including considerations in miRNA target identification and delivery.

Keywords Wound healing • HypoxymiRs • Angiogenesis • Inflammation • Re-epithelialization

Introduction

A chronic wound is defined as a wound or any interruption in the continuity of the body's surface that requires a prolonged time to heal (over 4 weeks), does not heal, or recurs. Examples of some chronic wounds are diabetic foot ulcers, venous ulcers, and pressure ulcers and constitute a serious clinical problem with high morbidity and mortality. Chronic wounds are common and constitute a significant health problem. It has been estimated that 1–2 % of the population of industrialized

J. Banerjee (✉)
Extremity Trauma and Regenerative Medicine Division, US Army Institute of Surgical Research,
3698 Chambers Pass, BHT-1, Joint Base-San Antonio, Fort Sam Houston, San Antonio, TX 78234, USA
e-mail: jaideep.b@gmail.com

C.K. Sen
The Ohio State University Wexner Medical Center, Davis Heart & Lung Research Institute, Comprehensive Wound Center, Center for Regenerative Medicine & Cell-Based Therapies,
473 West, 12th Avenue, Columbus, OH 43210, USA
e-mail: Chandan.Sen@osumc.edu

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_15

countries will experience a leg ulcer at some time [1]. In the USA alone, chronic wounds affect 6.5 million patients [2]. The immense economic and social impact of wounds in our society calls for enhancing our understanding of the biological mechanisms underlying cutaneous wound complications. Chronic wound care is expensive and therefore treatment options that are both clinically effective and cost-effective are vital.

miRNA Biogenesis and Mechanism of Action

microRNAs (miRs) are a class of ~21–23 nucleotide long noncoding RNAs (ncRNAs) that regulate the functionality of majority of protein-coding genes. Mature miRNAs are transcribed by RNA polymerase II as precursor RNAs, called primary miRNAs (pri-miRNAs) [3]. The pri-miRNAs are then sequentially processed in the nucleus and cytoplasm by the RNase III/Drosha/DGCR8 complex to hairpin-shaped pre-miRNAs (~70 nucleotides in length) that are further shortened in the cytoplasm by Dicer to give rise to the mature miRNA [3]. In the cytoplasm, miRNAs associate with mRNAs within the RISC complex (RNA-induced silencing complex), which facilitates and stabilizes miRNA–mRNA interactions. miRNAs anneal via Watson–Crick base pairing, with sequences most commonly located in 3′ untranslated regions (UTRs) of mRNA [3] although there are some examples of miRNA interactions within mRNA coding regions, intron–exon junctions, and 5′ UTRs [4, 5]. Association of a miRNA with its mRNA target results in degradation of the mRNA as well as inhibition of translation (Fig. 15.1).

microRNA-based gene silencing plays a critical role in the tissue repair response following wounding. The ability to therapeutically manipulate miRNA expression through systemic or local delivery of miRNA inducers/inhibitors has triggered enthusiasm about the therapeutic potential of miRNAs for non-healing wounds. An analysis from Frost & Sullivan has determined that US microRNA markets have earned revenues of over $20.3 million in 2008 and estimate this to reach $98.6 million by 2015 [6, 7]. microRNA research thus offers the capability of moving from bench to bedside faster than most research fields and diagnostic tests have already emerged from this young field.

OxymiRs in Wound Healing

microRNAs that are implicated in defining biological outcomes in response to a change in the state of tissue oxygenation are referred to as oxymiRs [8]. OxymiRs can be directly influenced by changes in tissue oxygenation or indirectly by oxygen-sensitive transcription factors, metabolites, pH, etc. or may also affect other oxygen-sensing pathways. miRNAs that are sensitive to hypoxia are classified as “hypoxymiRs”. HIF is one of the most important hypoxia sensors in our body and

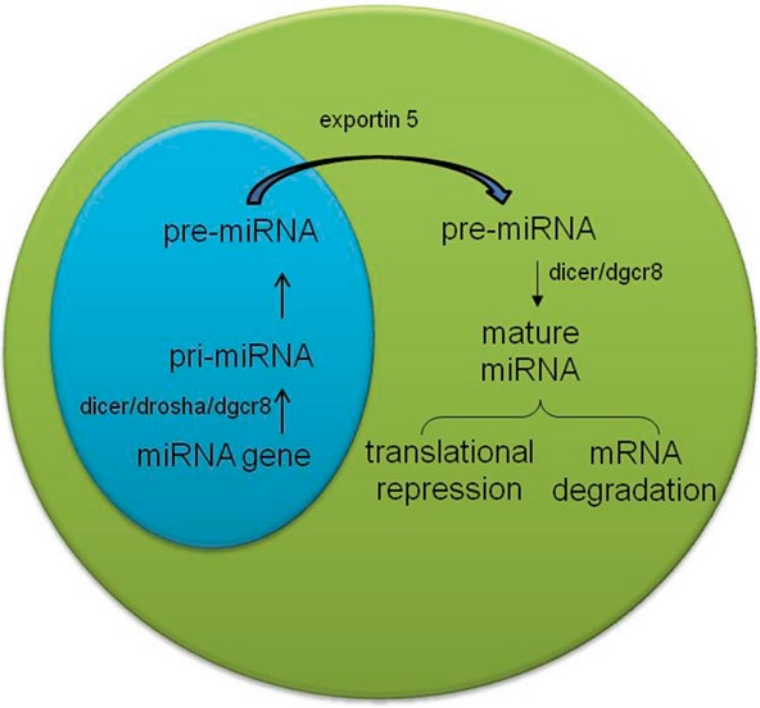

Fig. 15.1 Biogenesis of microRNAs

a number of hypoxymiRs are induced by HIF stabilization [9]. Some miRNAs also influence HIF stabilization. Induction of miR-424 is known to stabilize HIF1α, while miRs that target the HIF1α transcript such as miR-20b, miR-199a, and miR-17~92 are repressed to increase HIF1α expression and transcription [10, 11].

Chronic non-healing wounds are characterized by ischemia/hypoxia. HypoxymiRs have therefore been a strong focus to identify dysregulated targets for possible therapeutic intervention. miR-210, miR-21, and miR-203 are the most studied hypoxymiRs with respect to wound healing [12]. The key oxymiRs that are differentially expressed during the different stages of wound healing are as follows (Fig. 15.2):

miRNAs in Inflammation Control in Wound Healing

The first report linking miRNAs with inflammation came from miRNA expression profiling in a monocytic cell line treated with lipopolysaccharide (LPS) TLR4 ligand [13] which induced expression of miR-146a, miR-155, and miR-132 [13].

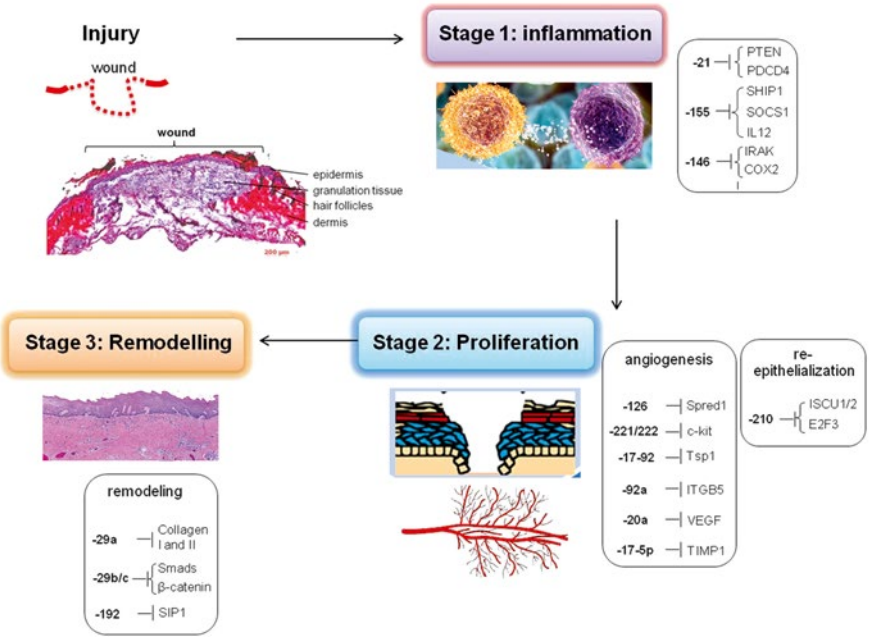

Fig. 15.2 MicroRNAs involved in different stages of wound healing

Inflammatory cells, including macrophages and neutrophils, recognize invading microbial pathogens primarily through Toll-like receptors (TLRs) [14]. miR-155 is among the first miRNAs to be linked to inflammation induced by TLRs ligands, inflammatory cytokines, and specific antigens. miR-155 regulates proteins involved in the cellular immune response against pathogens, which have clinical implication in chronic infected wounds [15]. miR-155 was identified to be directly repressing src homology-2 domain-containing inositol 5-phosphate 1(SHIP1) [16]. Furthermore, miR-155 indirectly enhances TNFα translation by its influence on LPS signaling mediators such as Fas-associated death domain protein (FADD), IκB kinase epsilon, and the receptor (TNFR superfamily)-interacting serine-threonine kinase 1 (Ripk1) [17]. LPS-induced downregulation of miR-125b is also instrumental in bolstering the production of TNF-alpha [18]. miR-125b has been shown to bind to the 3′-UTR of TNF-α inhibiting the translation of this cytokine [18]. In addition, the genes regulated by TNFα, i.e., E-selectin and ICAM-1 are direct targets of miR-31 and miR-17-3p, respectively [19]. Another major chemokine involved in wound healing is the chemokine macrophage chemo attractant protein (MCP-1/CCL2). It is a major chemo-attractant for monocytes/macrophages. The expression of MCP-1 was highly upregulated (~70 fold) following wounding [20]. miR-124a is directly implicated in the posttranscriptional silencing of MCP-1 [21].

IRAK1 and TRAF6 are two prominent targets of miR-146a that help it to negatively regulate the release of IL8 and RANTES [14]. IRAK2, which regulates IFN-γ, has also been identified as a miR-146a target [16]. Downregulation of miR-146a has been observed in diabetic mouse wounds, thereby increasing its pro-inflammatory target genes [18]. miR-146a also acts as a critical physiological brake to prevent the overactivation of the innate as well as the adaptive immune system [19].

At an injury site, efficient clearance of apoptotic cells by wound macrophages or efferocytosis is a prerequisite for the timely resolution of inflammation. Resolvin D1, an endogenous lipid mediator generated during resolution phase of acute inflammation, induces miR-21, and therefore miR-21 has been proposed to play a role in resolving acute inflammation [22]. miR-21 silences PTEN, GSK3β, and PDCD4, which are all key effectors of the inflammatory response [23]. Decreased PDCD4 additionally favors c-Jun-AP-1 activity, which in turn results in elevated production of anti-inflammatory IL-10 [23].

Involvement of Dicer and miRNAs in Barrier Function during Re-epithelialization

Under conditions of oxygen limitation, the respiratory chain is limited, by the unavailability of oxygen as the final electron acceptor. In order to support survival, cells switch from aerobic to anaerobic metabolism, first described by the *Pasteur Effect*. Poor oxygen availability trigger austerity measures aimed at energy conservation [24, 25]. Nonessential energy consuming functions are suspended to support cell survival. Hypoxia regulates gene expression through transcriptional and post-transcriptional mechanisms. The processes by which cells sense and respond to ambient oxygen concentration are fundamental to cell survival and function, and they commonly target gene regulatory events. Hypoxia can also differentially regulate microRNAs, which are posttranscriptional regulators of genes. While under hypoxia, some microRNAs can be transcriptionally induced, chronic hypoxia impairs Dicer (*DICER1*) expression and activity, resulting in global suppression of microRNA biogenesis. VHL-dependent downregulation of Dicer is also linked to the expression and function of HIF-1α [26].

Recently, we have also shown that following arrest of Dicer in keratinocytes, barrier function of the repaired skin is severely disrupted, thus affecting wound healing [27]. On the other hand, Argonaute 2 (Ago2), which processes some precursor miRNAs independent of Dicer, has been shown to be increased in hypoxia. Hydroxylation of Ago2, which occurs as a response to hypoxia, is required for the association of Ago2 with heat shock protein 90 (Hsp90), which is necessary for the loading of microRNAs (miRNAs) into the RISC, and translocation to stress granules (SGs) [28]. Lack of molecular oxygen or a dysfunctional mitochondria to process that oxygen, and the resulting differentially expressed gene regulators, thus have a profound importance in wound healing.

The state of tissue oxygenation is widely recognized as a major microenvironmental cue that determines healing outcomes [29]. Healthy mitochondria are the main site of oxygen metabolism, accounting for approximately 85–90 % of the oxygen consumed by the cell [30, 31]. Mitochondria constantly metabolize oxygen, thereby producing ROS as a by-product. In the 1980s, oxygen free radicals drew much attention in biomedical research. The primary identity of free radicals was that they were destructive to biological tissues, and that approaches to antagonize free radicals, i.e., antioxidants are helpful [32–34]. Based on this crude preliminary concept, numerous clinical trials testing the efficacy of antioxidants were hastily started and the results were understandably disappointing [29]. Work during the mid-late 1990s led to the recognition that at very low levels, oxygen-derived free radicals and derivative species such as H_2O_2 may serve as signaling messengers [35–37]. This underlines the importance of oxygen in driving molecular signals during wound healing.

Although hypoxia may have favorable effects on endothelial cells, HIF specifically induces growth arrest of keratinocytes compromising wound closure. HIF1α is widely known to be pro-angiogenic [38]. It would thus be tempting to propose that HIF1α would improve wound healing. Under moderate hypoxia conditions such as the non-ischemic (low lactate) wound, HIF1α seems to favor wound closure [39]. HIF has been widely known as a transcription factor that regulates the expression of several coding genes. miR-210 has recently emerged as a key noncoding gene that is transcriptionally driven by HIF adding a new dimension to HIF's circle of influence in molecular and cell biology.

So far, the connection between HIF and miR-210 has been studied in the context of cancer [40, 41]. Work in our laboratory links miR-210 as a key regulator of ischemic wound closure. miR-210 also represses mitochondrial respiration and associated downstream functions [42]. We noted that the hypoxamiR miR-210 silences target genes which would arrest growth as well as repress mitochondrial metabolism in keratinocytes. This could be of importance in the healing wound where metabolic demand on mitochondrial metabolism is known to be high. In both mice and chronic wound patients, ischemic wounds showed elevated miR-210 levels. Sharply elevated lactate is also a hallmark of the ischemic tissue, including wounds [43, 44]. In addition to serving metabolic needs, lactate triggers cell signaling [43, 45]. We and others have reported that lactate induces HIF-1α signaling [43, 46]. Here, we show that lactate induces miR-210. Lactate also enhanced HIF-dependent miR-210 expression suggesting the presence of HIF-independent component in lactate-induced miR-210 expression.

Next, we screened for miR-210-dependent pathways that would impair wound re-epithelialization. We studied the candidate pathways that emerged from murine studies for their relevance to human chronic wounds. Expression of E2F3 which is a known target of miR-210 [40] was significantly lower in ischemic wounds. Immunohistochemical studies identified abundant E2F3 in the epidermis of non-ischemic wound. E2F3 in the wound-edge tissue of ischemic wounds was markedly downregulated.

To test the significance of HIF-1 in regulating E2F3 and cell proliferation in keratinocytes, studies were performed using human HaCaT keratinocytes commonly used for the study of wound healing [47]. HIF-1α stabilization, adopting a genetic approach, resulted in attenuated expression of E2F3, an observation that was consistent with the results from in vivo studies suggesting that HIF-1 downregulates E2F3 via a miR-210-dependent pathway. Knockdown of E2F3 limited cell proliferation, demonstrating the significance of E2F3 in driving keratinocyte cell cycle. Human ischemic wounds also demonstrated lower E2F3 and Ki67 (a proliferation marker). miR-210 is also known to suppress ISCU1/2, a protein needed for mitochondrial function. We indeed found that in the ischemic wounds of both humans and mice, with elevated miR-210, energy supply (ATP/ADP ratio) is sharply lower, which was in accordance with dysfunctional mitochondria.

We also discovered another novel microRNA, miR-1, to be hypoxia inducible and highly elevated in murine as well as human ischemic wounds (unpublished data). We found that c-met, which is known to upregulate AQP3 [48], a protein important for keratinocyte migration [49] is a direct target for miR-1 in keratinocytes, and overexpressing miR-1 in keratinocytes can impair cell migration. miR-1 is also predicted to silence IGF-1, another key player for keratinocyte migration [50, 51].

Thus, miR-1- and miR-210-directed therapeutic strategies to address complications in ischemic wound closure may prove to be a prudent consideration (Fig. 15.3).

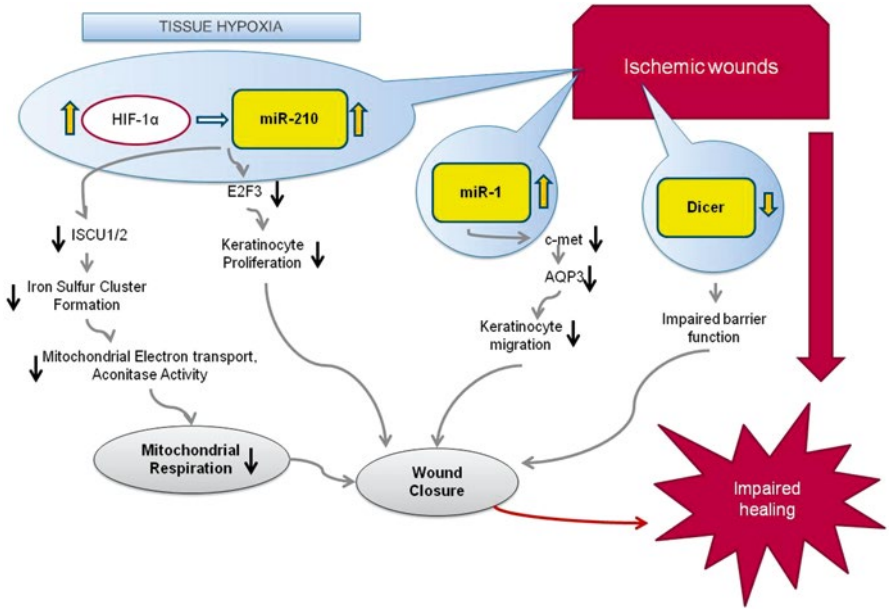

Fig. 15.3 Hypoxia-regulated microRNAs impairing wound re-epithelialization

miRNAs Involved in Angiogenic Response during Wound Healing

The clue to the importance of microRNAs in guiding vascularization was first obtained from experimental studies involved in arresting miRNA biogenesis by Dicer knockdown in vascular cells to deplete available mature miRNA pools [52, 53]. Dicer knockdown leads to profound dysregulation of angiogenesis-related genes [54]. Several aspects of angiogenesis, such as proliferation, migration, and morphogenesis, of endothelial cell are modified by specific miRNAs. Endothelial miRs involved in angiogenesis, also referred to as angiomiRs, include miR 17-5p, cluster 17–92, miR-15b, -16, -20, -21, -23a, -23b, -24, -27a, -29a, -30a, -30c, -31, -100, -103, -106, -125a and -b, -126, -181a, -191, -199a, -221, -222, -320, and let-7 family [55].

Dicer knockdown in human microvascular endothelial cells (HMECs) showed lower inducible production of ROS when activated with phorbol ester, TNFα, or VEGF. NADPH oxidase-derived reactive oxygen species (ROS) are important as signaling messengers in driving wound angiogenesis [56]. Transcription factor HBP1, a suppressor transcription factor that negatively regulates p47phox expression was also induced following Dicer knockdown. Knockdown of HBP1 restored the production of inducible ROS and the angiogenic response of miRNA-deficient HMECs [52].

Hypoxia is widely recognized as a cue that drives angiogenesis as part of an adaptive response to vascularize the oxygen-deficient host tissue. Figure 15.4 shows how microRNAs silence the pro-angiogenic effects of HIF-1. Hypoxia-repressible miR-200b is involved in induction of angiogenesis via directly targeting Ets-1 [57]. Certain Ets-1-associated genes, namely matrix metalloproteinase 1 and vascular endothelial growth factor receptor 2, were silenced by miR-200b. Overexpression of Ets-1 rescues miR-200b-dependent impairment in angiogenic response and suppression of Ets-1-associated gene expression [57]. VEFG and FGF-2 represent two key stimuli that drive wound angiogenesis in a concerted

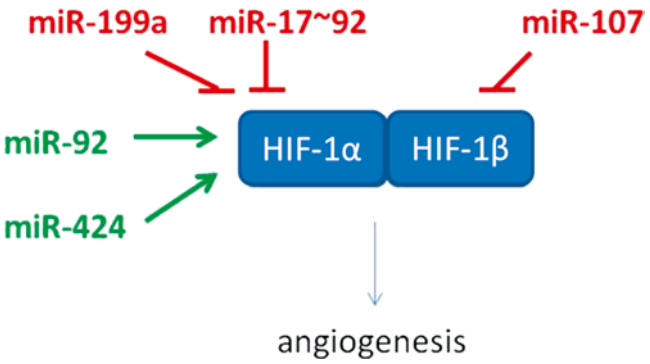

Fig. 15.4 Regulation of pro-angiogenic effect of HIF by microRNAs

manner. VEGF-A has been shown to induce the expression of miR-191, -155, -31, -17-5p, -18a, and miR-20a in HUVEC [58].

Both VEGF-A and basic FGF-2 increased the expression of miR-130a, a pro-angiogenic miRNA, which directly targets GAX and HOXA5 [59]. VEGF-A and bFGF signaling phosphorylate CREB causing rapid transcription of miR-132. miR-132 overexpression increased endothelial cell proliferation and in vitro networking by targeting p120RasGAP, a GTPase-activating protein [60]. miR-221 and miR-222 have been identified as modifying c-Kit expression as well as the angiogenic properties of the c-Kit ligand Stem Cell Factor. The miR-221/2 and c-Kit interaction represents an integral component of a complex circuit that controls the ability of endothelial cells to form new capillaries [61]. Inhibition of c-Kit results in reduced VEGF expression [62].

From Bench to Bedside: Therapeutic Applications of miRNAs

With the discovery of miRNAs beingpowerful regulators in wound healing and a wide variety of other diseases, the possibilities of therapeutically correcting the dysregulated miRNAs are being actively explored. miRNAs have several significant advantages in that they are small and comprise a known sequence that is often completely conserved among species, which are very attractive features from a drug development standpoint. Based on lessons learned from antisense technologies, very potent oligonucleotide chemistries to target miRNAs, known as anti-miRs or pharmacologically active synthetic miRNAs, or miR mimics, are currently being generated.

There are several significant advantages to miRNAs for becoming a new class of drug targets. Their small size and known and conserved sequence make them attractive candidates from a development standpoint. Additionally, many genetic or oligonucleotide-based gain-and-loss-of function studies have shown very pronounced phenotypes in rodents and even large animal models, whereas miRNA manipulation under baseline conditions oftentimes does not exert overt effects. Furthermore, the direct downstream targets of a single miRNA are commonly related genes that function in a comparable cellular process or signaling cascade. This implies that targeting of a single miRNA probably will result in a dramatic effect due to the combinatorial effect of gene expression changes in all these related downstream targets.

The impact of targeting a miRNA sequence is further strengthened by the fact that the genome often contains multiple copies of the same or closely related miRNA sequences, such that targeting of the miRNA sequence will become even more influential [63]. The biggest advantage of miR therapy besides having the ability to target multiple genes of a pathway at the same time is that plasma levels of an anti-miR or miR-mimetic are cleared from plasma within hours by uptake into tissues [63, 64] but, once inside cells, many of the modified oligonucleotide used as anti-miRs are so metabolically stable that their clearance is slow, and half-lives in tissues are often in the order of weeks, providing therapeutic benefit long after blood levels are

near zero. Because of their high water solubility, it is possible to dissolve anti-miRs in aqueous solutions at volumes that are amenable to self-administration by the subcutaneous route, and, with their long biological half-lives, it may be possible that anti-miRs can be administered relatively infrequently, thus reducing frequency of injection. miR therapy therefore presents an exciting potential for clinical application in the future.

Challenges in miRNA Therapeutics

Successful delivery of miRNA has some major challenges, which include low cellular uptake of the RNA and endosomal escape, immunogenicity, degradation in the bloodstream, and rapid renal clearance [65]. Delivery of the miRNA in order to be effective has to be routed to the target organ, enter the cell, and reach its intracellular target in an active form [65]. Therefore, new efforts are being made to develop more efficient methods to deliver miRNAs. Follow-up preclinical studies will have to guide appropriate dosing regimens in order to establish the lowest possible efficacious doses while attempting to prevent unacceptable side effects. A key limiting factor is the extremely poor efficiency of the internalization and release of anti-miRs [66] and therefore determining how to reach a sufficient dose within the cells in order to achieve efficient miRNA inhibition is a big challenge.

Considerations in Target Identification and miRNA Delivery

miRNAs often exhibit relatively modest inhibitory effects on mRNA targets. It is not uncommon, for example, for miRNA inhibition to result in minimal increases (<1.5–2-fold) in the expression of mRNA targets, suggesting that it is the cumulative impact of small changes in the expression of myriad targets—rather than pronounced changes in single targets—that mediates the biological actions of miRNAs on disease processes. Thus, when identifying targets, it may be beneficial to manipulate miRNAs that target multiple genes of a pathway that is dysregulated in a pathological condition.

Successful delivery of RNAi molecules in vivo is based mainly on incorporation into lipid- or polymer-based nanoparticles. An ideal delivery system should be biocompatible, biodegradable or excretable, and nonimmunogenic [65]. Natural and synthetic lipids and polymers such as phosphatidylcholine, PLGA, and chatoyant, which can undergo biodegradation into products absorbed by the natural biochemical pathways of the body, are the most common nanocarriers for therapeutics delivery.

Other requirements of the delivery system include being stable in the circulation, arriving at the target site, facilitating cellular uptake, avoiding lysosomal degradation, enabling endosomal escape, and bypassing rapid renal clearance [67]. miRNA

molecules are negatively charged and hydrophilic, therefore, incorporation of the negatively charged nucleic acid with cationic lipids or polymers resulting in a net positive charge enables it to cross the negatively charged cell membranes by receptor mediated endocytosis or pinocytosis. The nanoparticle complex (<100 nm in diameter) is coated with targeting molecules that can specifically interact with the target antigen on the cell surface. The outer surface of the nanoparticle contains hydrophilic groups in order to avoid rapid clearance by the reticuloendothelial system and enhance circulation time when injected into the bloodstream.

The nanocarriers then escape from the early endosome in order to avoid fusion with the lysosome and ultimately elimination via the Golgi system. The nonviral miRNA delivery by nanocarriers can generally be divided into three main categories: complexation, encapsulation, and conjugation. Complexation is the formation of an electrostatic complex between the negatively charged miRNA and the positively charged vehicle. Liposomes which are lipid vesicles composed of bilayer phospholipid membrane are the best examples. Encapsulation inside biodegradable nanoparticles such as PLGA or silica nanoparticles is another approach for miRNA delivery. The cargo is released following intracellular dissolution of the particle. This method is advantageous since it does not require the use of potentially toxic cationic materials.

Conjugation is the process of covalently binding the miRNA to its carrier using different linkers that will release the cargo specifically at the target site either by hydrolysis or reduction. Conjugation results in a highly stable delivery system that can effectively protect miRNA in the bloodstream [65]. Three forms of chemically modified oligonucleotides that have been used are (a) 2′-O-methyl-group (OMe)-modified oligonucleotides; (b) 2′-O-Methoxyethyl-modified oligonucleotides; and (c) Locked nucleic acid or LNA [68]. Another approach involves linking a RNA-binding protein or domain to the Fab fragments of a cell or tissue-specific antibody. The high affinity and binding specificity of antibodies make them attractive vehicles for targeted delivery of miR mimics or anti-miRs in vivo. Other considerations that need to be factored in, when designing the delivery system, include the knowledge of the tissue architecture, the microenvironment, and therapeutically meaningful doses that is required for efficient miRNA inhibition. Designing a delivery system that is more efficient in releasing the miR mimics or anti-miRs from the endosomes will considerably decrease the therapeutic dose of the oligonucleotides and improve therapeutic potential (Fig. 15.5).

Conclusion

The induction and silencing of a unique set of genes represent a trigger for the onset of pathology and restoration of the normal expression levels is key to the repair process. This well-orchestrated symphony is under the control of the small nucleotide regulators in the body called microRNAs. In this chapter, we have reviewed the miRs that have been identifying to be dysregulated during the different stages of the healing cascade.

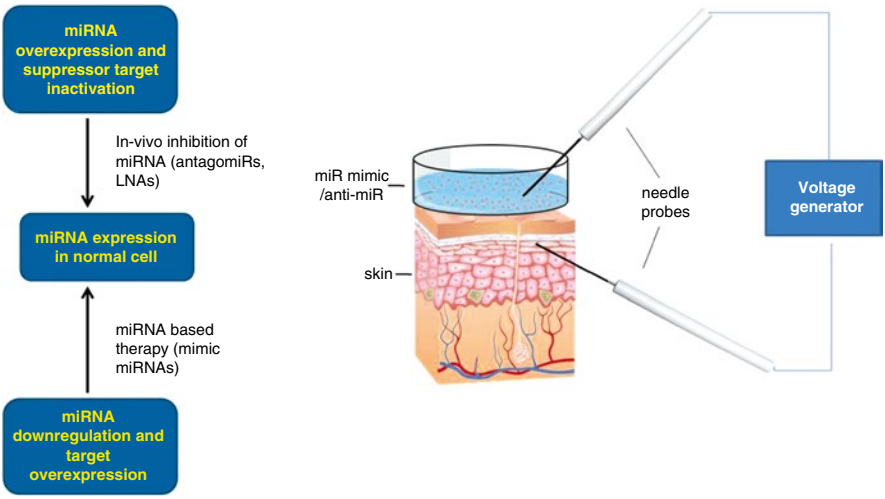

Fig. 15.5 Strategies of miR manipulation. Electroporation device for nanodelivery of miR manipulators

As newer miRs and targets for these miRs are discovered, the therapeutic prospect will improve for use of miR manipulators in skin injury repair. microRNA-210, which is induced highly in ischemic conditions, is already being tested in a clinical trial as molecular marker that can be used to predict healing outcomes, thereby making treatments more accurate and efficient. Further development of the technology into a successful therapy will involve (a) identification of new miRNA targets and dissecting their function; (b) identifying and improving agents capable of successful in vivo delivery of the antagomiR; and (c) delivering the nucleotides to the specific targeted sites. Thus, along with identifying new microRNA targets, future investigations need to be directed towards more efficient in vivo delivery systems.

Acknowledgement Supported by NIH RO1 grants GM069589, GM077185, and NR013898 to Chandan K Sen and DK076566 to Sashwati Roy.

References

1. Gottrup F. A specialized wound-healing center concept: importance of a multidisciplinary department structure and surgical treatment facilities in the treatment of chronic wounds. Am J Surg. 2004;187(5A):38S–43.
2. Sen CK, et al. Human skin wounds: a major and snowballing threat to public health and the economy. Wound Repair Regen. 2009;17(6):763–71.
3. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2): 215–33.

4. Tay Y, et al. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature. 2008;455(7216):1124–8.
5. Orom UA, Nielsen FC, Lund AH. MicroRNA-10a binds the 5′UTR of ribosomal protein mRNAs and enhances their translation. Mol Cell. 2008;30(4):460–71.
6. Ravishankar D. Global analysis of microRNA tools and services market: evolving microRNA market provides an opportunity for vendors of qRT-PCR and functional tools. Frost & Sullivan Research Service. Sep 2003.
7. Analysis of microRNA tools and services market in Europe: microRNA research triggers tremendous growth in tools market. Frost & Sullivan Research Service. Feb 2012.
8. Sen CK, Roy S. OxymiRs in cutaneous development, wound repair and regeneration. Semin Cell Dev Biol. 2012;23(9):971–80.
9. Loscalzo J. The cellular response to hypoxia: tuning the system with microRNAs. J Clin Invest. 2010;120(11):3815–7.
10. Cascio S, et al. miR-20b modulates VEGF expression by targeting HIF-1 alpha and STAT3 in MCF-7 breast cancer cells. J Cell Physiol. 2010;224(1):242–9.
11. Rane S, et al. Downregulation of miR-199a derepresses hypoxia-inducible factor-1alpha and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. Circ Res. 2009;104(7):879–86.
12. Kulshreshtha R, et al. A microRNA component of the hypoxic response. Cell Death Differ. 2008;15(4):667–71.
13. Taganov KD, et al. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A. 2006; 103(33):12481–6.
14. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004;4(7):499–511.
15. Martinez-Nunez RT, et al. MicroRNA-155 modulates the pathogen binding ability of dendritic cells (DCs) by down-regulation of DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN). J Biol Chem. 2009;284(24):16334–42.
16. O'Connell RM, et al. Inositol phosphatase SHIP1 is a primary target of miR-155. Proc Natl Acad Sci U S A. 2009;106(17):7113–8.
17. Tili E, Croce CM, Michaille JJ. miR-155: on the crosstalk between inflammation and cancer. Int Rev Immunol. 2009;28(5):264–84.
18. Tili E, et al. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol. 2007;179(8):5082–9.
19. Suarez Y, et al. Cutting edge: TNF-induced microRNAs regulate TNF-induced expression of E-selectin and intercellular adhesion molecule-1 on human endothelial cells: feedback control of inflammation. J Immunol. 2010;184(1):21–5.
20. Roy S, et al. Characterization of the acute temporal changes in excisional murine cutaneous wound inflammation by screening of the wound-edge transcriptome. Physiol Genomics. 2008;34(2):162–84.
21. Nakamachi Y, et al. MicroRNA-124a is a key regulator of proliferation and monocyte chemoattractant protein 1 secretion in fibroblast-like synoviocytes from patients with rheumatoid arthritis. Arthritis Rheum. 2009;60(5):1294–304.
22. Recchiuti A, et al. MicroRNAs in resolution of acute inflammation: identification of novel resolvin D1-miRNA circuits. FASEB J. 2011;25(2):544–60.
23. Das A, et al. Engulfment of apoptotic cells by macrophages: a role of microRNA-21 in the resolution of wound inflammation. J Immunol. 2014;192(3):1120–9.
24. Aragones J, et al. Oxygen sensors at the crossroad of metabolism. Cell Metab. 2009;9(1): 11–22.
25. Boutilier RG, St-Pierre J. Surviving hypoxia without really dying. Comp Biochem Physiol A Mol Integr Physiol. 2000;126(4):481–90.
26. Ho JJ, et al. Functional importance of Dicer protein in the adaptive cellular response to hypoxia. J Biol Chem. 2012;287(34):29003–20.

27. Ghatak S, Chan YC, Khanna S, Banerjee J, Weist J, Roy S, Sen CK. Barrier function of the repaired skin is disrupted following arrest of Dicer in keratinocytes. Mol Ther. 2015;23(7): 1201–10.
28. Wu C, et al. Hypoxia potentiates microRNA-mediated gene silencing through posttranslational modification of Argonaute2. Mol Cell Biol. 2011;31(23):4760–74.
29. Sen CK. Wound healing essentials: let there be oxygen. Wound Repair Regen. 2009;17(1): 1–18.
30. Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. Physiol Rev. 1979;59(3):527–605.
31. Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci U S A. 1994;91(23):10771–8.
32. Clark IA, Cowden WB, Hunt NH. Free radical-induced pathology. Med Res Rev. 1985;5(3):297–332.
33. Dormandy TL. Free-radical pathology and medicine. A review. J R Coll Physicians Lond. 1989;23(4):221–7.
34. Slater TF. Free-radical mechanisms in tissue injury. Biochem J. 1984;222(1):1–15.
35. Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. FASEB J. 1996; 10(7):709–20.
36. Sen CK. Redox signaling and the emerging therapeutic potential of thiol antioxidants. Biochem Pharmacol. 1998;55(11):1747–58.
37. Sen CK. Cellular thiols and redox-regulated signal transduction. Curr Top Cell Regul. 2000;36:1–30.
38. Chen L, Endler A, Shibasaki F. Hypoxia and angiogenesis: regulation of hypoxia-inducible factors via novel binding factors. Exp Mol Med. 2009;41(12):849–57.
39. Sarkar K, et al. Tie2-dependent knockout of HIF-1 impairs burn wound vascularization and homing of bone marrow-derived angiogenic cells. Cardiovasc Res. 2012;93(1):162–9.
40. Giannakakis A, et al. miR-210 links hypoxia with cell cycle regulation and is deleted in human epithelial ovarian cancer. Cancer Biol Ther. 2008;7(2):255–64.
41. Huang X, et al. Hypoxia-inducible mir-210 regulates normoxic gene expression involved in tumor initiation. Mol Cell. 2009;35(6):856–67.
42. Chan SY, et al. MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2. Cell Metab. 2009;10(4):273–84.
43. Hunt TK, et al. Aerobically derived lactate stimulates revascularization and tissue repair via redox mechanisms. Antioxid Redox Signal. 2007;9(8):1115–24.
44. Loffler M, et al. Wound fluid lactate concentration: a helpful marker for diagnosing soft-tissue infection in diabetic foot ulcers? Preliminary findings. Diabet Med. 2011;28(2):175–8.
45. Hashimoto T, et al. Lactate sensitive transcription factor network in L6 cells: activation of MCT1 and mitochondrial biogenesis. FASEB J. 2007;21(10):2602–12.
46. Lu H, Forbes RA, Verma A. Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J Biol Chem. 2002;277(26):23111–5.
47. Sen CK, et al. Oxidant-induced vascular endothelial growth factor expression in human keratinocytes and cutaneous wound healing. J Biol Chem. 2002;277(36):33284–90.
48. Wang J, et al. c-Met upregulates aquaporin 3 expression in human gastric carcinoma cells via the ERK signalling pathway. Cancer Lett. 2012;319(1):109–17.
49. Levin MH, Verkman AS. Aquaporin-3-dependent cell migration and proliferation during corneal re-epithelialization. Invest Ophthalmol Vis Sci. 2006;47(10):4365–72.
50. Elia L, et al. Reciprocal regulation of microRNA-1 and insulin-like growth factor-1 signal transduction cascade in cardiac and skeletal muscle in physiological and pathological conditions. Circulation. 2009;120(23):2377–85.
51. Haase I, et al. Regulation of keratinocyte shape, migration and wound epithelialization by IGF-1- and EGF-dependent signalling pathways. J Cell Sci. 2003;116(Pt 15):3227–38.
52. Shilo S, et al. Evidence for the involvement of miRNA in redox regulated angiogenic response of human microvascular endothelial cells. Arterioscler Thromb Vasc Biol. 2008;28(3):471–7.

53. Kuehbacher A, et al. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. Circ Res. 2007;101(1):59–68.
54. Suarez Y, et al. Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. Circ Res. 2007;100(8):1164–73.
55. Caporali A, Emanueli C. MicroRNA regulation in angiogenesis. Vascul Pharmacol. 2011;55(4):79–86.
56. Sen CK, et al. Oxygen, oxidants, and antioxidants in wound healing: an emerging paradigm. Ann N Y Acad Sci. 2002;957:239–49.
57. Chan YC, et al. miR-200b targets Ets-1 and is down-regulated by hypoxia to induce angiogenic response of endothelial cells. J Biol Chem. 2011;286(3):2047–56.
58. Suarez Y, et al. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. Proc Natl Acad Sci U S A. 2008;105(37):14082–7.
59. Chen Y, Gorski DH. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. Blood. 2008;111(3):1217–26.
60. Anand S, et al. MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis. Nat Med. 2010;16(8):909–14.
61. Poliseno L, et al. MicroRNAs modulate the angiogenic properties of HUVECs. Blood. 2006;108(9):3068–71.
62. Litz J, Krystal GW. Imatinib inhibits c-Kit-induced hypoxia-inducible factor-1alpha activity and vascular endothelial growth factor expression in small cell lung cancer cells. Mol Cancer Ther. 2006;5(6):1415–22.
63. Levin AA. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. Biochim Biophys Acta. 1999;1489(1):69–84.
64. Geary RS, Yu RZ, Levin AA. Pharmacokinetics of phosphorothioate antisense oligodeoxynucleotides. Curr Opin Investig Drugs. 2001;2(4):562–73.
65. Ben-Shushan D, et al. Overcoming obstacles in microRNA delivery towards improved cancer therapy. Drug Deliv Transl Res. 2014;4(1):38–49.
66. Gilleron J, et al. Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. Nat Biotechnol. 2013;31(7):638–46.
67. Henry JC, Azevedo-Pouly AC, Schmittgen TD. MicroRNA replacement therapy for cancer. Pharm Res. 2011;28(12):3030–42.
68. Weiler J, Hunziker J, Hall J. Anti-miRNA oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease? Gene Ther. 2006;13(6):496–502.

Chapter 16
Essential Role of microRNA in Skin Physiology and Disease

Damjan Glavač and Metka Ravnik-Glavač

Abstract The identification and characterization of microRNAs (miRNAs) is a rapidly growing area of research also in dermatology. Skin represents the largest organ in the human body, and its morphogenesis has been shown to require a highly coordinated and undisrupted miRNA profile. High expression of several miRNAs in the epidermis and hair follicles is necessary for normal skin development. Profiling studies have identified numerous differentially regulated miRNAs associated with either normal physiological status of the skin or some pathological processes or both. This chapter covers current knowledge of the important roles of miRNAs in the pathogenesis of some skin diseases including systemic lupus erythematosus (SLE), systemic sclerosis (SSc), dermatomyositis (DM), psoriasis (PS), and skin cancer, especially malignant melanoma (MM). In addition, the diagnostic and therapeutic relevance of miRNAs that are involved in pathological processes of the skin are elucidated providing further information for some possible clinical implications especially for their use as therapeutic targets or disease biomarkers.

Keywords Skin diseases • Psoriasis • Malignant melanoma

Introduction

The skin is an essential and the largest organ in our body; it is metabolically active and regenerative through our life. It comprises about 15 % of the total body weight and is the body's primary defense structure. Skin has three layers: the epidermis, the

D. Glavač (✉)
Department of Molecular Genetics, Faculty of Medicine, University of Ljubljana, Korytkova 2, Ljubljana 1000, Slovenia
e-mail: damjan.glavac@mf.uni-lj.si

M. Ravnik-Glavač
Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, Ljubljana 1000, Slovenia

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_16

dermis, and hypodermis. The composition and thickness of the epidermis and dermis vary from 0.04 to 1.6 mm depending on the location on the body. The primary cell in the epidermis is the keratinocyte. Additional cells include the melanocytes (cells of neural crest origin that produce pigment), the Langerhans cells (mobile, dendritic antigen-presenting cells), and the Merkel cells (cells with both neuroendocrine and epithelial features that synapse with the dermal sensory axons and adjacent epithelial cells and are sensitive to mechanical stimuli [1].

The dermis, located between the subcutaneous and dermal–epidermal junction, measures from 0.3 mm on the eyelids to 3.0 mm on the back. The dermis and its blood supply are responsible for delivering nutrients and circulatory support. Communication is ongoing between the dermis and epidermis. The skin's color is created by melanocytes producing the pigment melanin and they are located in the epidermis. Functionally, the skin has the biggest challenge of any organ of the body. It is exposed and responds to environmental challenges. Every minute around 30,000 of our outermost skin cells die. The skin is also a highly active biological factory for protein synthesis and metabolism, signaling molecules and lipids, and an integral part of the immune, nervous, and endocrine systems [2].

Skin maintenance is the primary therapy for wound prevention: a wound is defined as the absence of healthy, normally developed, and intact skin. However, all of the functions of the skin change with age. The skin is therefore a complicated structure with many functions. Skin aging and failure, not usually visible processes, become evident when the skin is affected. In the failing individual, skin deterioration is the outward manifestation of faltering physiology. Even in the healthy aged population, normal and expected changes occur, leading to many problems [3]. Recent advances in miRNA research and technology in skin have provided evidence of the importance of miRNAs in normal cellular skin function and also in skin diseases.

miRNA Technology

High-throughput miRNA gene expression analysis is a technical challenge. The short length and uniqueness of each miRNA render many conventional tools ineffective; very small RNAs are difficult to reliably amplify or label without introducing bias. Earlier attempts at detection and identification of miRNA included three approaches: hybridization-based methods (e.g., Northern blot), PCR-based detection, and cloning methods. Based on these initial approaches, higher-throughput technologies have been developed systematically to profile miRNA on a genome-wide scale [4]. Three main methods are currently used to measure the expression levels of miRNAs: real-time reverse transcription-PCR (qPCR) [5, 6], microarray hybridization [7, 8], and massively parallel/next-generation sequencing (NGS) [9]. Each of these methods has its unique challenges for use in miRNA profiling. Microarray analysis is challenged by differentiating between mature and precursor miRNAs and differentiating among different miRNAs that differ by only one or a few nucleotides. Furthermore, the target preparation in itself is challenging due to

the fact that miRNAs are relatively short; in addition, there are limitations in probe designing and hybridization conditions to account for the unequal Tm of mature miRNAs [7]. NGS is increasingly popular because it allows for the discovery of new miRNAs together with quantitative expression analysis. As a result, the continued refinement of miRNA databases necessitates frequent changes to miRNA array probe design and annotation. However, NGS has its limitations as miRNAs can be influenced by sequencing errors and often require search and removal of adaptor sequences before the miRNA sequence itself can be elucidated [10]. In addition, both the RNA ligation [11] and the PCR amplification steps bear inherent biases and the method is costly; associated tools for computational analysis are still in developing stages [10].

It was shown also that miRNA profiling with)NGS is strongly biased toward certain small RNAs, which makes NGS inappropriate for absolute quantification of miRNAs, but not for differential miRNA expression analysis [12]. These biases were dependent on library preparation and were also observed in quantitative reverse transcription polymerase chain reaction (qPCR) amplifications [13]. Rapid increase in the number of miRNAs renders qPCR inefficient on a genomic scale, and it is probably better used as a validation rather than a discovery tool [10].

Microarray-based techniques have the advantages of being relatively cost-effective, relatively quick from RNA labeling to data generation, and simple to use. The differences between available platforms range from surface chemistry and printing technology, through probe design and labeling techniques, to cost. Among the available commercial miRNA microarray platforms, the single-color array format is the most common, but miRNA profiling platform technology has also been developed that provides both sequence and size discrimination for mature miRNAs [14].

In the studies comparing miRNA profiling results using different methodologies including different microarray platforms, qPCR and NGS, it was shown that the platform which uses direct labelling methods is probably less prone to amplification biases than PCR-based labelling methods, indicating that the technology type is not the major variability factor. It is likely that other factors, such as microarray probe design, target preparation, or hybridisation stringency are more important [10, 13].

miRNAs and Normal Skin Development

High expression of several miRNAs in the epidermis and hair follicles is necessary for normal skin development. The global importance of miRNAs in skin development was revealed in functional studies on animal models by targeting critical components in the miRNA biogenesis pathways such as Drosha-DGCR8 or Dicer. Mutants of Dicer and Dgcr8 conditional knockouts present similar phenotypes of severe defects in murine embryonic skin development. Both mutants have been characterised by rough skin, failure to thrive, defects in hair follicle slow-growth, and apoptosis [15]. Because the epidermis and hair follicles are accessible and easy to manipulate genetically, they also represent a good system to investigate the

functions of miRNA. In 2006 Yi and coworkers showed that many skin miRNAs are differentially expressed in the epidermis and hair follicles. They have cloned more than 100 miRNAs from skin and they have found 33 highly expressed miRNAs (>1 % abundance) in epidermis with three of them miR-200a, miR-141, and miR-429 being absent in hair follicles. Another 15 miRNAs were more abundant in epidermis than hair follicles. Interestingly, specific to only the hair follicle there were six abundant miRNAs, including the miR-199a family [16].

This early research on skin miRNA forms a basis to establish precisely the function of individual miRNAs in the skin. miRNA-203 has been identified as a skin-specific microRNA which was also the first skin miRNA thoroughly investigated. Expression of miR-203 depends on the stages of skin development and is cell specific. Overexpression of miR-203 in keratinocytes enhances keratinocyte differentiation. miRNA-203 has several validated targets [17]. One of those targets was p63 identified as a regulator of stem-cell maintenance in stratified epithelial tissue. miR-203 represses the expression of p63 and at least in part acts as a switch between proliferation and differentiation. Highly expressed miR-203 in the suprabasal layer has been shown to repress the expression of p63, thus reducing the proliferative potential of terminally differentiating keratinocytes [18].

In addition to miR-203, recent studies have also recognised several other miRNAs involved in skin development and homeostasis. For example, repression of miR-34 transcription by p63 is important to maintain cell cycle progression in epidermal cells [19]. miR-125b has been shown to be highly expressed in skin stem cells, in contrast to dramatically lower expression in their early progeny [20]. The miR-200 family and miR-205 are both highly expressed in normal skin. These miRNAs have been shown to target ZEB1 and ZEB2, which are both transcriptional repressors of E-cadherin [21]. On the other hand it was shown that downregulation of the miR-200 family and miR-205 induces an epithelial-to-mesenchymal transition in conjunction with upregulation of ZEB1 and ZEB2 [22].

In recent years NGS high-throughput sequencing technology was used for profiling the expression of various RNA species on a genomewide scale with single-nucleotide resolution. Recent NGS studies have indicated that more than 200 novel loci in the human genome were identified as hosting novel miRNAs and miRNA candidates, some of them also of particular interest in skin conditions such as psoriasis [23].

miRNAs in Skin Diseases

Many studies address the question of the pivotal role of miRNAs in the pathogenesis of various human disorders including skin diseases. First, miRNAs are important in the regulation of development, especially in regulating the timing of morphogenesis and the maintenance of undifferentiated or incompletely differentiated cell types (stem cell differentiation, cardiac and skeletal muscle development, neurogenesis, haematopoiesis, etc.). Second, they are involved in several

physiological processes such as insulin secretion, cholesterol metabolism, and immune response. Third, miRNAs have been linked to the formation of tumours and abnormal regulation of the cell cycle, where they can function as both oncogenes and tumour suppressors. Fourth, in addition to abnormal miRNA expression profiles in diseased in comparison to healthy tissues, disruptions of miRNA target interaction in the form of single-nucleotide polymorphisms (SNPs) either in the miRNA gene or its target site (3′ UTR mRNA) could lead to complete gain or loss of miRNA function, thus also leading to a disease state [24].

The role of miRNA in autoimmune diseases (AIMDs) is of special importance as skin lesions are an early manifestation in many of those conditions. AIMDs arise from an abnormal immune predisposition and environmental exposure. Given that miRNAs play key roles also in the regulation of the immune system, it is not surprising that several studies revealed links between miRNA dysfunction and AIMDs with skin involvement. They include among many others also systemic lupus erythematosus (SLE), systemic sclerosis (SSc), dermatomyositis (DM), and most notably, psoriasis (PS).

miRNAs in Psoriasis

Psoriasis is a common, chronic, immune-mediated systemic disease characterised by skin lesions. The causes of psoriasis are not fully understood. Psoriasis is an organ-specific autoimmune disease and the most prevalent chronic inflammatory skin disease, affecting 1–3 % of the population. Genetic and environmental factors in connection to abnormal regulation of the immune system are thought to be involved in pathogenesis of the disease. Several different cell types, keratinocytes, fibroblasts, monocyte-derived immune cells, T cells, and mast cells, which normally occur in skin, are relevant in the formation of psoriatic lesions [25]. Keratinocytes show abnormal differentiation and proliferation because of aberrant cell signalling and production of mediators involved in immune cell activation. It is widely accepted that psoriasis is a consequence of impaired crosstalk between the immune system and the structural cells of the skin [26]. To show a specific miRNA expression profile in psoriasis-affected skin, representing leukocyte- and keratinocyte-derived miRNAs, Sonkoly and coworkers compared psoriasis-affected skin to atopic eczema and to healthy human skin and identified miR-203, along with miR-146a, miR-21, and miR-125b, as a psoriasis-specific miRNA [27]. They also observed downregulation of an evolutionary conserved SOCS3 protein. This protein is involved in inflammatory responses and keratinocyte proliferation and differentiation. In response to IL-6, which is a cytokine present in the psoriatic lesions, STAT3 transcription factor is activated, which leads to development of psoriatic plaques [28]. SOCS3, target of miR-203, functions also as an inhibitor of the activation of STAT3. It is suggested that the SOCS3 suppression by miR-203 in psoriatic lesions would lead to constant activation of STAT3, subsequent infiltration of immune cells, and the development of psoriatic plaques [27].

Identified psoriasis-associated miRNAs in the skin showed different expression profiles among 21 studied organs. miR-203 was expressed more than 100-fold higher in skin compared to most other organs, and at lower levels in organs with squamous epithelium, suggesting a specific function for this miRNA in the formation/function of squamous epithelia. miR-146a, miR-21, and miR-125b were detected in all studied organs, but their expression showed different patterns. miR-146a was highly expressed in organs containing a high percentage of leukocytes with lower expression in healthy skin, suggesting that infiltrating cells express miR-146a in the skin. miR-125b was expressed mostly in organs containing cells of ectodermal origin. miR-21 showed the highest expression in the bladder, lung, prostate, and colon [27].

Systematic analyses of expression of identified psoriasis-associated miRNAs in the skin revealed a list of microRNA listed in Table 16.1 and Fig. 16.1. TNF-alpha is one of the most important mediators in leukocyte–keratinocyte interactions in psoriasis [26]. Psoriasis-specific miRNA, miR-146a, inhibits the expression of two

Table 16.1 Summary of involvements of miRNAs and miRNAs putative targets in the pathogenesis of psoriasis [27, 30–35]

miRNA	Putative targets	Cell type	Location	Regulation
miR-19a	CCND1, KIT	Hair roots	13q31.3	Up-regulated
miR-21	BTG2, PDCD4, RASGRP1, RPS7, TIMP3, SOX5, RECK, TGFBR2, PTEN, TPM1, APAF1, BMPR2, MSH2, MTAP	Dendritic and mast cells	17q23.1	Up-regulated
miR-31	STK40, DKK1, DACT3	Keratinocytes	9p21.3	Up-regulated
miR-99a	mTOR, IGF1R, FGFR3	Skin	21q21.1	Down-regulated
miR-125b (miR-125b-1, miR-125b-2)	ERBB2, CBFB, TP53INP1, KLF13	Fibroblasts	11q24.1, 21q21.1	Down-regulated
miR-146a	CXCR4, TLR4, TRAF6, IRAK1, BRCA1, NFKB1, SMAD4	Dendritic and mast cells	5q34	Up-regulated
miR-150	MYB, EGR2	Skin	19q13.33	Down-regulated
miR-197	IL22RA1	Keratinocytes	1p13.3	Down-regulated
miR-203	SOCS-3, p63, TNFα, IL-24	Keratinocytes	14q32.33	Up-regulated
miR-423	CDKN1A	Skin	17q11.2	Unchanged
miR-424	MEK1, HIF1A, CUL2, SPI1, CCNE1, CCND3, CDK6, CCND1, MAP2K1, MYB, SIAH1	Keratinocytes	Xq26.3	Down-regulated

There are several web-based computational miRNA target gene prediction programs including: miRTarBase (http://mirtarbase.mbc.nctu.edu.tw), PicTar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org/)

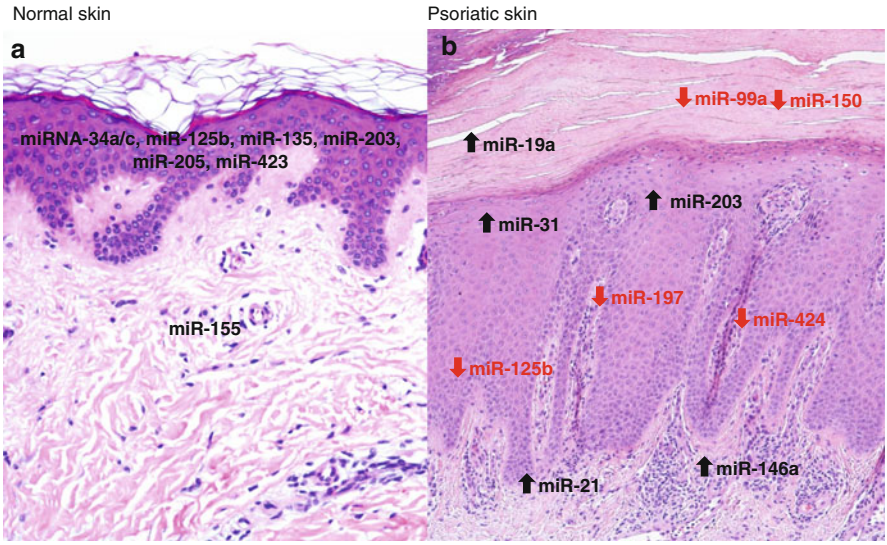

Fig. 16.1 miRNAs in normal skin (**a**) and psoriatic skin (**b**): five most prominent downregulated miRNAs (in *red*) and five most prominent upregulated miRNAs (in *black*)

regulators of the TNF-a signalling pathway, IRAK1 and TRAF6. Thus, miRNA-146a is probably involved in the pathogenesis of psoriasis via the modulation of TNF-alpha signalling in the skin [27, 29].

miRNAs in Systemic Lupus Erythematosus, Systemic Sclerosis, and Dermatomyositis

Dai and coworkers compared the levels of miRNAexpression in SLE patients with idiopathic thrombocytopenic purpura (ITP) patients using miRNA microarrays. They found 22 SLE- and ITP-related miRNAs, of which 13 miRNAs had the same expression pattern in both SLE and ITP and might be generally associated with AIMDs. Three miRNAs were SLE-related and may be involved in systemic inflammation; the other 6 ITP-related miRNAs may have functions in organ-specific destruction of thrombocytes. Thus, SLE-specific and ITP-specific miRNAs are potential biomarkers for differential diagnosis of SLE and ITP and are probably involved in pathogenesis of the SLE and ITP [36]. In another microRNA-SLE related study miRNA-142-3p and miRNA-142-5p were significantly downregulated in SLE CD4+ T cells compared with normal healthy controls [37].

Lower expression of miRNA-146a has been found to be negatively correlated with SLE disease activity. At the molecular level, miRNA-146a targets IRF5 and STAT1 which are important transcription factors related to the interferon signalling network [38]. Decreased miRNA-145 and increased miRNA-224 expression in T

cells from patients with SLE were also reported [39]. The aberrant production of cytokines and chemokines in SLE T cells are also mediated by the abnormal downregulation of miRNAs. T cells from SLE patients produce remarkably low amounts of IL-2 upon activation. A recent report has demonstrated that the significant reduction in miRNA-31 expression in SLE T cells is positively correlated with the lowered IL-2 production [40].

Type I IFN is recognised as the central player in SLE pathogenesis from the striking correlation between the deregulated type I IFN-inducible gene expression pattern and SLE disease activity, also known as the "IFN signature" of SLE [41]. There are fewer studies on miRNA expression in B cells in patients with SLE. Liu and colleagues have reported a direct interaction between miRNA-30a and the 3′-UTR of Lyn mRNA in association with deregulated B cell functions in SLE patients [42]. Three miRNA polymorphisms miRNA-499 rs3746444, miRNA-146a rs57095329, and miRNA-146a rs2910164, were investigated in patients with rheumatoid arthritis (RA) or SLE using a meta-analysis approach. The results demonstrate that miRNA-499 rs3746444 polymorphism has a significant association with RA but not with SLE, miRNA-146a rs57095329 has a significant association with SLE, and miRNA-146a rs2910164 has no association with either RA or SLE [43]. Collectively, there is a lack of distinct pattern in specific deregulated miRNA expression in SLE among the reviewed studies. Further studies with a larger sample size are needed to evaluate the association of miRNA with SLE precisely.

The expression pattern of multiple miRNAs in individual patients with scleroderma with that in normal subjects was also compared and significant difference observed. Levels of let-7g and miR-125b showed strong correlation in normal subjects, but not in patients with scleroderma. The miRNA expression pattern of combined miR-206 and miR-21 was useful to distinguish patients with scleroderma [44]. The possibility to use miRNA-29a levels in hair as a disease marker of patients with scleroderma was also evaluated. Comparison of miR-29a levels in scleroderma patients, dermatomyositis patients, and normal controls revealed lower levels of hair miRNA-29a in scleroderma patients than those in control subjects or dermatomyositis, suggesting that hair miR-29a microRNAs may serve as an independent biomarker [45]. Downregulation of miR-29a is thought to mediate the posttranscriptional upregulation of collagen, which contributes to the tissue fibrosis in scleroderma [46]. In another similar study serum and hair root of miRNA-196a levels were not significantly changed in scleroderma patients, whereas miRNA-196a levels in hair shafts were significantly decreased in scleroderma patients compared to those in normal subjects [47].

The levels of extracellular circulating miRNA levels have been detected in several skin diseases in which objective biomarkers have not been in clinical use and they are listed in Table 16.2. In dermatomyositis patients, for example, serum miRNA-21 levels were significantly upregulated and correlated with serum IgG levels whereas serum miRNA-223 and miRNA-7 were downregulated [48, 49]. In psoriatic patients a statistically significant difference in the serum levels of miR-125b, miR-146a, miR-203, and miR-205 among normal subjects and psoriasis patients was reported [50]. In scleroderma patients serum miRNA-150 levels were decreased and those patients tended to have more severe clinical manifestations [51].

Table 16.2 Summary of miRNA, miRNAs putative targets, and miRNAs expression in serum of patients with skin diseases [27, 35, 50–56]

miRNA	Putative targets	Disease	Location	Regulation
miR-7 (miR-7-1, miR-7-2, miR-7-3)	EGFR, RAF1, SNCA	Dermatomyositis	9q21.32, 15q26.1, 19p13.3	Down-regulated
miR-21	BTG2, PDCD4, RASGRP1, RPS7, TIMP3, SOX5, RECK, TGFBR2, PTEN, TPM1, APAF1, BMPR2, MSH2, MTAP	Psoriasis, Dermatomyositis	17q23.1	Down regulated in psoriasis; up-regulated in dermatomyositis
miR-24 (miR-24-1, miR-24-2)	CDKN1B, FEN1, CDK4, CCNA2, AURKB, MYC, E2F2, DHFR, HNF4A, BRCA1, POLD1, Smad3, CDK1	Psoriasis	9q22.32, 19p13.13	Unchanged
miR-29a	SPARC, DNMT3A, MCL1, PPM1D, CDK6, SERPINB9	Scleroderma	7q32.3	Unchanged, decreased in scleroderma spectrum disorder
miR-125b (miR-125b-1, miR-125b-2)	ERBB2, CBFB, TP53INP1, KLF13	Psoriasis	11q24.1, 21q21.1	Down-regulated
miR-143	KRAS, MAPK7, MYO6, DNMT3A, FNDC3B, JAG1	Psoriasis	5q32	Up-regulated
miR-146a	CXCR4, TLR4, TRAF6, IRAK1, BRCA1, NFKB1, SMAD4	Psoriasis	5q34	Down-regulated
miR-150	MYB, EGR2, MUC4, ZEB1	Scleroderma	19q13.33	Down-regulated

(continued)

Table 16.2 (continued)

miRNA	Putative targets	Disease	Location	Regulation
miR-196a (miR-196a1, miR-196a2)	HOXC8, ANXA1, HOXB7, BACH1, HMOX1	Scleroderma	17q21.32, 12q13.13	No change
miR-203	SOCS-3, p63, TNFα, IL-24	Psoriasis	14q32.33	Down-regulated in serum (up-regulated in keratinocytes)
miR-205	ZEB1, ERBB3, ZEB2, EGLN2	Psoriasis	1q32.2	Down-regulated
miR-223	NFIA, MEF2C, STMN1, LMO2, RHOB, CHUK, PARP1	Psoriasis, Dermatomyositis	Xq12	Up-regulated in psoriasis, down-regulated in dermatomyositis
miR-424	MEK1, HIF1A, CUL2, SPI1, CCNE1, CCND3, CDK6, CCND1, MAP2K1, MYB, SIAH1	Psoriasis	Xq26.3	No change in serum (down-regulated in keratinocytes)

miRNAs and Malignant Melanoma

Malignant melanoma is the most aggressive form of skin cancer and, according to the World Health Organization (WHO), the number of melanoma cases worldwide is increasing faster than that of any other type of cancer. Melanoma accounts for only about 4 % of skin cancer cases but for as many as 74 % of all skin cancer deaths. Although there is a good chance for the recovery of patients suffering from melanoma, if the primary lesion is detected very early (more than 90 % survival in stage I melanomas), prognosis of 5-year survival for more advanced melanomas drops down across approximately 60 % (stage II) of cases to as low as 10 % (stage III) or to even complete fatality (stage IV). This reflects the current lack of therapeutic approaches for treating advanced melanoma [57].

That miRNA profiles were suitable to classify melanoma with respect to their developmental lineage and differentiation state was shown first in the study of Lu et al. [58]. Further studies included more melanoma and other cancer cell lines analysing miRNA copy number or miRNA expression patterns [59, 60]. They identified a set of 15 miRNAs that were expressed significantly differently in the eight melanoma cell lines [60]. In 2009, more comprehensive studies were published [61, 62], carrying out a detailed comparison of the miRnomes of normal human melanocytes to well-characterised melanoma cell lines derived from primary tumours and melanoma metastases. It has been shown that several miRNAs are associated with

malignant transformation as well as with different steps of melanoma progression, including invasion and metastasis [61]. In order to determine miRNAs which were deregulated in the transition of melanocytes into melanoma cells, Mueller and coworkers compared expression of 461 miRNAs in melanocytes with primary melanoma cell lines. They detected 77 upregulated and only 14 downregulated miRNAs in primary melanoma cell lines. Of these 49 miRNAs were upregulated more than tenfold in primary melanoma cell lines or were not expressed in melanocytes but highly expressed in primary melanoma cell lines thus strongly associated with early progression. Comparing miRNA expression levels in primary melanoma cell lines and melanoma cell lines derived from metastatic melanomas revealed 13 differently regulated miRNAs associated with metastatic colonisation [61]. Using real-time PCR to determine the expression level of 16 potentially relevant miRNAs of 6 preparations of normal melanocytes versus 10 melanoma cell lines and in formalin fixed paraffin embedded tissue of 11 melanocytic nevi versus 16 melanomas, miR-15b and miR-210 were significantly upregulated, whereas miR-34a was significantly downregulated in melanomas compared with melanocytic nevi. These 3 miRNAs were further analysed in a total of 128 primary melanomas from patients with detailed clinical follow-up information. High expression of miR-15b was significantly associated with poor recurrence-free survival and overall survival. Downregulation of miR-15b in two melanoma cell lines with high miR-15b expression by transfection with anti-miR-15b siRNA was associated with reduced tumour cell proliferation, whereas apoptosis was increased. MiR-15b thus appeared to represent a particular important miRNA in melanoma that is associated with poor prognosis and tumourigenesis [62].

Melanoma cell lines and patient tissue samples including benign nevi and primary and metastatic tumours were analysed, in order to compare their miRNA expression pattern with those of the cell lines. Using miRNA microarrays and real-time miRNA reverse transcription-PCR arrays, followed by qPCR validation Philippidou et al., 2010 detected inconsistent results, both between different detection techniques as well as between tissue samples and cell lines. Nevertheless, they identified miRNA-200c to be consistently downregulated in melanocytes, melanoma cell lines, and patient samples, whereas miRNA-205 and miRNA-23b were markedly reduced only in patient samples [63].

In a recent study [64] an exploratory microarray analysis was performed by miRNA expression profiling based on platform screening for 1205 human miRNAs using specimens excised from the center of tumours from patients with primary cutaneous malignant melanoma (PCMM), cutaneous malignant melanoma metastases, and benign melanocytic tissue obtained during surgery. The results from the microarray analysis were validated by TaqMan quantitative real-time polymerase chain reaction. They confirmed significantly increased expression of several miRNAs associated with PCMM development including hsa-miR-106, hsa-miR-126, hsa-miR-133a, hsa-miR-141, hsa-miR-145, hsa-miR-15b, hsa-miR-200c, hsa-miR-210, and hsa-miR-27b [61, 62, 65, 66] as well as those significantly downregulated that have been repeatedly described in other studies and include miRNAs hsa let-7a, hsa-let-7b, hsa-miR-155, hsa-miR-324-5p, and hsa-miR-34a [61, 62, 65, 67,

68]. In addition to the above-mentioned miRNAs previously known to be associated with cutaneous malignant melanoma (CMM), Sand et al. 2013 [64] reported additional novel candidate miRNAs to be deregulated in CMM patient samples. Among them hsa-miR- 22, hsa-miR-130b, hsa-miR-146b-5p, hsa-miR-223, hsa-miR-301a, hsa-miR-484, hsa-miR-663, hsa-miR-720, hsa-miR-1260, hsa-miR-3663-3p, hsa-miR-4281, and hsa-miR-4286 were found to be upregulated, and hsa-miR-24-1*, hsa-miR-26a, hsa-miR-4291, hsa-miR-4317, and hsa-miR-4324 were downregulated . Of these hsa-miR-130b and hsa-miR-26a were also confirmed in a study using next-generation sequencing methodology [69].

The majority of studies has shown miRNAs deregulated in melanoma using mostly cell lines [61, 70–75] and fewer studies using specimens of primary tumours [63, 76–78] or melanoma metastases [79]. In addition, these studies were based on microarray hybridisation or real-time quantitative RT-PCR (qRT-PCR) techniques, which are limited to the detection of known miRNA sequences previously identified by sequencing or homology searches.

NGS sequencing is a promising alternative technology as it is unable to identify and quantify also novel miRNAs. Stark and coworkers deep sequenced small RNA libraries from melanoblasts, melanocytes, and congenital nevi as well as from acral, mucosal, cutaneous, and uveal melanoma cells, and identified 539 known mature miRNAs along with 279 predicted novel miRNAs. Some of the potentially novel miRNAs were likely to be specific for the melanocytic lineage and might become interesting candidates in melanoma research [80].

In a recent study Kozubek and coworkers used deep sequencing to characterise the miRNA transcriptome in melanoma [69]. They compared miRNA profiles of biopsies of nevi, thick primary melanomas, and metastatic melanomas with matched normal skin in parallel to melanocytes and melanoma cell lines (both primary and metastatic) and identified 698 differently expressed miRNAs. In the set of 40 miRNAs that were most statistically significantly differentially expressed in all samples they confirmed 23 (58 %) previously discovered miRNAs (including miR-15b, miR-205, miR-23b, miR-126, miR-200c, hsa-let-7a, hsa-let-7b), introduced an additional 17 (42 %) known and top-15 putative novel candidate miRNAs deregulated during melanoma progression. To classify benign from malignant lesions, they performed an unsupervised clustering on all the samples and showed four major subgroups with an almost-perfect separation between normal and cancer. Downregulation of miR-144-3p, miR-181b-5p, miR-320a, miR-320c, miR-320d, and miR-451a separated melanoma from normal skin, and downregulation of miR-203, miR-204-5p, miR-205-5p, miR-211-5p, miR-23b-3p, miR-26a-5p, and miR-26b-5p distinguished melanoma from nevi. Moreover, specific miRNA signature distinguished primary melanomas from metastatic lesions. Higher levels of miR-103/107 clusters in primary melanoma were associated with the clinical history of early occult metastasis to sentinel lymph node and miR-205 was significantly decreased in primary melanoma and metastases to lymph node compared to nevi [69]. miR-205 was shown also to be significantly associated with worse clinical outcome [81].

Kozubek et al. also detected a significant divergence in miRNA signature between melanoma specimens and low-passage–number cell lines highlighting the inherent epigenetic/genetic differences between human malignancies and their cultured counterparts. Inasmuch as most studies have profiled miRNAs from established melanoma cell lines compared to cultured melanocytes they further discussed the possibility of making these miRNA results clinically irrelevant [69].

Functional Role of miRNAs in Malignant Melanoma

Because of the complexity of molecular mechanisms influenced by miRNA activity and the heterogeneous experimental approaches, a complete picture of the role of miRNAs in malignant melanoma is still missing [82].

miRNAs have been shown to be frequently located at fragile sites or at genomic sites that are often altered in cancer. Some specific miRNAs that have been shown to be underexpressed, mapped to regions that are frequently deleted or to be overexpressed and located at regions frequently amplified in cancers. Each miRNA can control hundreds of gene targets and thus potentially influence almost every genetic pathway. Recent evidence has shown that miRNA mutations or mis-expression correlate with various human cancers and indicates that miRNAs can act as either tumour suppressors or oncogenes, depending on their downstream target genes [83]. Generally, miRNAs in melanoma intersect with cycle/proliferation pathways, cell invasion pathways, and cell survival pathways as well as modulate immune response [79] (Fig. 16.2). Most functionally characterised miRNAs detected as deregulated in melanoma tissues and confirmed by different studies using different methodologies and different techniques include let-7b, miR-200 family, miR-203, miR-211-5p, miR-204-5p, miR-205-5p, miR-23b-3p, miR-26a-5p, and miR-26b-5p as downregulated and miR-221/211, miR-21, miR-182, miR-30b/30d¸ miR-214, and miR-148b as upregulated.

Downregulated miRNAs in Melanoma

Let-7b expression was significantly reduced in melanoma compared to nevi, suggesting a role of let-7b in the transition from nevi to primary melanomas. The 3′UTRs of the human RAS genes contain multiple let-7 complementary sites, allowing let-7 to regulate RAS expression [84]. Predicted targets of let-7 family miRNAs include N-RAS, RAF, c-Myc, cyclins D1, D3, and A, and Cdk4, all of which had been shown to play a role in melanoma biology [68, 85–87]. These studies demonstrated that let-7 family members act as tumour suppressors whose loss may contribute to melanoma development and progression through the activation of various oncogenic processes including signalling pathways acting on proliferation, migration, and invasion.

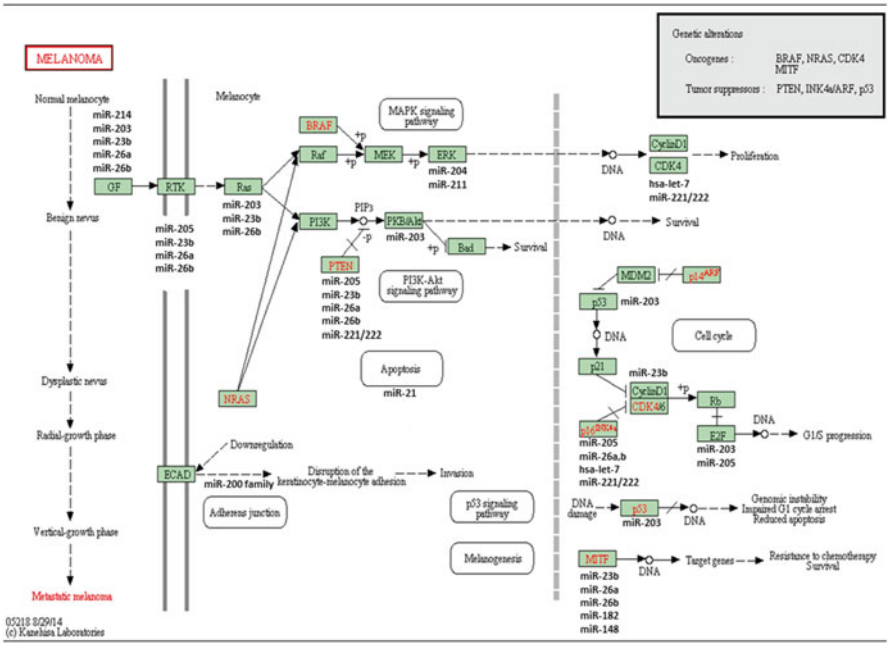

Fig. 16.2 miRNAs in melanoma cycle/proliferation pathways. http://www.kegg.jp/kegg/kegg1.html

Recently, it was described that miR-26a inhibited tumour growth and metastasis formation and upregulated let-7 [88, 89]. miR-26a was broadly reduced in multiple cancers, and overexpression of miR-26a significantly suppressed tumour growth and metastasis both in vitro and in vivo, including melanoma, prostate, and liver cancers. It was shown also that one miRNA can act as a modulator of another miRNA biogenesis, because miR-26a directly targeted Lin28B and Zcchc11, two critical repressors of let-7 maturation [90].

In both, primary and metastatic melanoma, compared with benign melanocytic nevi, miRNAs of the miR-200 family, including miR-200a, miR-200b, miR-200c, and miR-141 were shown to be downregulated during melanomagenesis and are therefore suggested as tumour suppressors [77]. Overexpression of miR-200c was reported to result in a significant decrease of proliferation, migration, and drug resistance in melanoma caused by miR-200c–mediated downregulation of BIM-1 [91]. Loss of miR-200c may also contribute to the release of repression of ZEB1, a transcription factor that represses E-cadherin transcription, a major prerequisite for epithelial–mesenchymal transition [92]. miR-141 showed an additional decrease in melanoma metastases compared to primary melanoma [77].

In addition, miR-203, miR-211-5p, miR-204-5p, miR-205-5p, miR-23b-3p, miR-26a-5p, and miR-26b-5p were downregulated in primary cutaneous melanoma versus common nevi. Examining a specific KEGG pathway by downregulation of these miRNAs in melanoma highlighted the mitogen-activated protein kinase

(MAPK) signalling pathway. Within this pathway, often more than one miRNA is predicted to target FGF18, PDGFRA, PIK3R3, PTEN, AKT2, and MAPK1, where the same gene could be potentially targeted by several cotranscribed miRNAs. For example, miR-205, miR-23b, miR-26a, and miR-26b converge on PDGFRA or miR-211 and miR-204 converge on MAPK1, demonstrating a combinatorial effect of miRNAs on the same target [69].

Upregulated miRNAs in Melanoma

Reports that the expression of the receptor tyrosine kinase c-KIT was found to decrease with melanoma progression [93, 94] and that miR-221/222 target c-KIT in papillary thyroid carcinoma [95] and in erythroleukemic cells [96] influenced Felicetti et al. to analyse the expression of miR-221/222 and c-KIT in melanocytes and melanoma cell lines including primary vertical growth phase and metastatic melanomas. They found that miR-221/222 expression increased with tumour progression and reversely correlated with c-KIT expression. They also showed that downregulation of PLZF in melanoma cells unblocks miR-221/222 which in turn represses the expression of cyclin-dependent kinase inhibitor 1B (CDKN1B) and c-KIT, leading to increased proliferation and inhibited differentiation, respectively. Further in vivo experiments showed that overexpression of miR-221/222 promoted tumour growth whereas inhibition of miR-221/222 by respective antagomirs suppressed tumour growth [97]. It was shown also that miR-221 and 222, by targeting PTEN and TIMP3 tumour suppressors, induce TNF-related apoptosis-inducing ligand (TRAIL) resistance and enhance cellular migration through the activation of the AKT pathway and metallopeptidases. It was also demonstrated that the MET oncogene is involved in miR-221 and 222 activation through the c-Jun transcription factor [98]. In a recent study c-FOS was identified as another direct target of miR-221 and 222 whose repression causes reduced apoptosis [99].

It was demonstrated in a recent study that levels of miR-21 were significantly increased in primary melanoma tissues as compared to benign nevi [100]. In a previous research Talotta et al. have found that the tumour suppressors PTEN and PDCD4 are downregulated by RAS in an AP-1 and miR-21 dependent fashion [101]. In melanoma cell lines with high endogenous miR-21 expression downregulation of miR-21 induced apoptosis, whereas proliferation was not significantly altered.

miR-182 is frequently amplified in melanoma and was found to be upregulated in melanoma cell lines and tissues [102]. MITF and FOXO3 were shown to be direct targets of miR-182. In human tissues, expression of miR-182 increases with progression from primary to metastatic melanoma and inversely correlates with FOXO3 and MITF levels. Silencing of miR-182 led to apoptosis in melanoma cells and diminished their invasive capacity in vitro [102].

Upregulated miR-30b/30d expression was described to correlate with melanoma stage, metastatic potential, shorter time to recurrence, and reduced overall survival. Ectopic expression of miR-30b/30d promoted the metastatic behaviour of mela-

noma cells by directly targeting the GalNAc transferase GALNT7, resulted in increased synthesis of the immunosuppressive cytokine IL-10, and reduced immune cell activation and recruitment [60]. Similar oncogenic effects could also be observed for miR-214, a driver of melanoma metastases [61]. Upregulation of miR-214 and ALCAM and the loss of TFAP2 expression have been implicated in this process, with TFAP2 a direct target of miR-214. miR-214 upregulated ALCAM, acting transcriptionally through TFAP2 and also posttranscriptionally through miR-148b (itself controlled by TFAP2), both negative regulators of ALCAM. A pathway involving miR-214, miR-148b, TFAP2, and ALCAM was thus defined which regulates melanoma cell migration, extravasation, invasion, and angiogenesis resulting in establishing distant metastases in melanoma [62].

MiRNAs as Diagnostic and Prognostic Biomarkers in Melanoma

Because of lineage-specific expression of some miRNAs, miRNAs have the ability to subclassify tumour types more specifically than mRNAs. Furthermore, miRNAs are important modulators of classical oncogenes and tumour suppressors, which turn them into putative tumour suppressors or oncogenes. miRNAs are as well extremely stable molecules, both in archived tissues and body fluids, a property which, in combination with their tissue specificity, makes them attractive candidate biomarkers [103].

The remarkable stability of miRNAs in the bloodstream [104] and the ability of miRNAs to distinguish between disease-free and disease-burdened patients offered new hope of miRNA use in early detection or disease surveillance. Leidinger et al. [105] identified a miRNA signature of melanoma comparing the miRNA profiles of blood cells from patients with melanoma ($n=35$) versus healthy donors ($n=20$). They detected 16 significantly deregulated miRNAs (miR-186, let-7d, miR-18a, miR-145, miR-99a, miR-664, miR-501-5p, miR-378, miR-29c, miR-1280, miR-365, miR-1249, miR-328, miR-422a, miR-30d, miR-17) that could separate the groups with a classification accuracy of 97.4 %, a specificity of 95 %, and a sensitivity of 98.9 % by supervised analysis [105]. These results are particularly promising because most of the patients included in the analysis were diagnosed with early-stage melanoma, suggesting the significant potential of miRNA expression sensitivity to early disease changes and detection in blood cells.

In tissue samples different mi-RNAs signatures, namely downregulation of miR-144-3p, miR-181b-5p, miR-320a, miR-320c, miR-320d, and miR-451a separated melanoma from normal skin [69].

miRNAs may also have powerful prognostic potential in melanoma. Patient melanoma specimens expressing lower levels of miRNA-205 by immunohistochemistry have been shown to associate tightly with significantly shorter melanoma-specific survival, independent of melanoma stage, age, gender, or Breslow depth [81]. In vitro and in vivo models have demonstrated that miR-205 overexpression impedes melanoma cell migration and invasion [91].

Another miRNA, miR-29c, was demonstrated to be significantly downregulated in AJCC stage IV melanoma specimens compared to primary tumours. Elevated expression of miR-29c significantly predicted disease-free and overall survival [106].

Friedman and coworkers [107] performed a miRNA regression analysis comparing serum samples from patients with melanoma against serum samples from patients with other cancers, systemic inflammatory disease, and healthy volunteers. They found that miR-182 and miR-221 are specific for melanoma compared with all three controls and that miR-182, miR-221, and miR-15b are predictive for metastasis. A signature of five miRNAs (miR-150, miR-15b, miR-199a-5p, miR-33a, miR-424) predicted high versus low recurrent-free survival groups [107]. Higher expression of a signature of 18 miRNA: miR-150, miR-455-3p, miR-145, miR-342-3p, miR-497, miR-155, miR-342-5p, miR-143, miR-193a-3p, miR-146-5p, miR-28-3p, miR-10b, miR-193b, miR-28-5p, miR-143, miR-126, and miR-214 was significantly correlated with longer survival in melanoma patients with metastasis [79].

miRNAs in Nonmelanoma Skin Cancer

miRNAs in Basal Cell Carcinoma

Basal cell carcinoma (BCC) is the most common form of human cancer, which rarely metastasises [108]. In advanced cases, tumour growth can spread to types of tissue other than skin, such as cartilage, bone, nerves, and vessels [109].

In a study by Heffelfinger et al. [110] a global miRNA expression was quantified by high-throughput sequencing in nodular BCCs, a subtype that is slow growing, and infiltrative BCCs, aggressive tumours that extend through the dermis and invade structures such as cutaneous nerves. Twenty miRNAs showed statistically significant differences in expression between nodular and infiltrative BCCs, including miR-150, miR-31, miR-183, miR-146a, and miR-886-5p, which were validated also by qPCR. The expression level of miR-183, a miRNA that inhibits invasion and metastasis in several types of malignancies, was consistently lower in infiltrative than nodular tumours and could be one element underlying the difference in invasiveness [110]. In a microarray-based miRNA expression profile study of tumour biopsies of 7 patients with BCC of the skin compared to the adjacent nonlesional skin 16 significantly upregulated (hsa-miR-17, hsa-miR-18a, hsa-miR-18b, hsa-miR-19b, hsa-miR-19b-1*, hsa-miR-93, hsa-miR-106b, hsa-miR-125a-5p, hsa-miR-130a, hsa-miR-181c, hsa-miR-181c*, hsa-miR-181d, hsa-miR-182, hsa-miR-455-3p, hsa-miR-455-5p, and hsa-miR-542-5p) and 10 significantly downregulated (hsa-miR-29c, hsa-miR-29c*, hsa-miR-139-5p, hsa-miR-140-3p, hsa-miR-145, hsa-miR-378, hsa-miR-572, hsa-miR-638, hsa-miR-2861, and hsa-miR-3196) miRNAs were identified. Data mining revealed connections to many tumour-promoting pathways, such as the Hedgehog and the MAPK/extracellular signal-regulated kinase signalling cascades [111].

miRNAs in Cutaneous Squamous Cell Carcinoma

Cutaneous squamous cell carcinoma (cSCC)is an epithelial skin tumour that is the second most common form of human cancer [108]. Some cSCCs become locally invasive and show an aggressive course [112]. The rate of metastasis has been shown to be 0.3–3.7 % with an overall 5-year survival rate <30 % in cases in which it spreads systemically [113].

Microarray miRNA profiling of tumour biopsies from patients with cSCC and adjacent healthy skin revealed three upregulated (hsa-miR-135b, hsa-miR-424, and hsa-miR-766) and six downregulated (hsa-miR-30a*, hsa-miR-378, hsa-miR-145, hsa-miR-140-3p, hsa-miR-30a, and hsa-miR-26a) miRNAs in cSCC [114].

Conclusion

The aberrant expression and function of miRNAs has been linked to the development and progression of many human diseases, including skin diseases. SLE, SSc, dermatomyositis, psoriasis, and skin cancer, especially malignant melanoma, are skin conditions which were intensively studied in recent years using miRNA technology. Some skin disease related specific miRNA and miRNAs signatures have been identified and a few miRNA pathways elucidated. In the future more functional studies involving antagomirs will eventually lead to the confirmation of present studies and identification of new diagnostic and prognostic markers as well as therapeutic targets to be tested in preclinical models.

References

1. McGrath JA, Eady RAJ, Pope FM. Anatomy and organization of human skin. In: Rook A, Burns T, editors. Rook's textbook of dermatology. Malden: Blackwell; 2004. p. 3.1–3.84.
2. Fore J. A review of skin and the effects of aging on skin structure and function. Ostomy Wound Manage. 2006;52:24–35; quiz 36–27.
3. Giacomoni PU, Declercq L, Hellemans L, Maes D. Aging of human skin: review of a mechanistic model and first experimental data. IUBMB Life. 2000;49:259–63.
4. Sun BK, Tsao H. Small RNAs in development and disease. J Am Acad Dermatol. 2008;59:725–37; quiz 738–740.
5. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005;33, e179.
6. Shi R, Chiang VL. Facile means for quantifying microRNA expression by real-time PCR. Biotechniques. 2005;39:519–25.
7. Yin JQ, Zhao RC, Morris KV. Profiling microRNA expression with microarrays. Trends Biotechnol. 2008;26:70–6.
8. Li W, Ruan K. MicroRNA detection by microarray. Anal Bioanal Chem. 2009;394: 1117–24.

9. Hafner M, Landgraf P, Ludwig J, Rice A, Ojo T, Lin C, Holoch D, Lim C, Tuschl T. Identification of microRNAs and other small regulatory RNAs using cDNA library sequencing. Methods. 2008;44:3–12.
10. Git A, Dvinge H, Salmon-Divon M, Osborne M, Kutter C, Hadfield J, Bertone P, Caldas C. Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. RNA. 2010;16: 991–1006.
11. Bissels U, Wild S, Tomiuk S, Holste A, Hafner M, Tuschl T, Bosio A. Absolute quantification of microRNAs by using a universal reference. RNA. 2009;15:2375–84.
12. Linsen SE, de Wit E, Janssens G, Heater S, Chapman L, Parkin RK, Fritz B, Wyman SK, de Bruijn E, Voest EE, Kuersten S, Tewari M, Cuppen E. Limitations and possibilities of small RNA digital gene expression profiling. Nat Methods. 2009;6:474–6.
13. Pradervand S, Weber J, Lemoine F, Consales F, Paillusson A, Dupasquier M, Thomas J, Richter H, Kaessmann H, Beaudoing E, Hagenbuchle O, Harshman K. Concordance among digital gene expression, microarrays, and qPCR when measuring differential expression of microRNAs. Biotechniques. 2010;48:219–22.
14. Wang H, Ach RA, Curry B. Direct and sensitive miRNA profiling from low-input total RNA. RNA. 2007;13:151–9.
15. Xia J, Zhang W. MicroRNAs in normal and psoriatic skin. Physiol Genomics. 2014;46:113–22.
16. Yi R, O'Carroll D, Pasolli HA, Zhang Z, Dietrich FS, Tarakhovsky A, Fuchs E. Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. Nat Genet. 2006;38:356–62.
17. Yi R, Poy MN, Stoffel M, Fuchs E. A skin microRNA promotes differentiation by repressing 'stemness'. Nature. 2008;452:225–9.
18. Lena AM, Shalom-Feuerstein R, Rivetti di Val Cervo P, Aberdam D, Knight RA, Melino G, Candi E. miR-203 represses 'stemness' by repressing DeltaNp63. Cell Death Differ. 2008;15:1187–95.
19. Antonini D, Russo MT, De Rosa L, Gorrese M, Del Vecchio L, Missero C. Transcriptional repression of miR-34 family contributes to p63-mediated cell cycle progression in epidermal cells. J Invest Dermatol. 2010;130:1249–57.
20. Zhang L, Stokes N, Polak L, Fuchs E. Specific microRNAs are preferentially expressed by skin stem cells to balance self-renewal and early lineage commitment. Cell Stem Cell. 2011;8:294–308.
21. Korpal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J Biol Chem. 2008;283:14910–4.
22. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008;10:593–601.
23. Joyce CE, Zhou X, Xia J, Ryan C, Thrash B, Menter A, Zhang W, Bowcock AM. Deep sequencing of small RNAs from human skin reveals major alterations in the psoriasis miRNAome. Hum Mol Genet. 2011;20:4025–40.
24. Bostjancic E, Glavac D. Importance of microRNAs in skin morphogenesis and diseases. Acta Dermatovenerol Alp Pannonica Adriat. 2008;17:95–102.
25. Menter A, Gottlieb A, Feldman SR, Van Voorhees AS, Leonardi CL, Gordon KB, Lebwohl M, Koo JY, Elmets CA, Korman NJ, Beutner KR, Bhushan R. Guidelines of care for the management of psoriasis and psoriatic arthritis: section 1. Overview of psoriasis and guidelines of care for the treatment of psoriasis with biologics. J Am Acad Dermatol. 2008;58:826–50.
26. Lowes MA, Bowcock AM, Krueger JG. Pathogenesis and therapy of psoriasis. Nature. 2007;445:866–73.

27. Sonkoly E, Wei T, Janson PC, Saaf A, Lundeberg L, Tengvall-Linder M, Norstedt G, Alenius H, Homey B, Scheynius A, Stahle M, Pivarcsi A. MicroRNAs: novel regulators involved in the pathogenesis of psoriasis? PLoS One. 2007;2, e610.
28. Croker BA, Krebs DL, Zhang JG, Wormald S, Willson TA, Stanley EG, Robb L, Greenhalgh CJ, Forster I, Clausen BE, Nicola NA, Metcalf D, Hilton DJ, Roberts AW, Alexander WS. SOCS3 negatively regulates IL-6 signaling in vivo. Nat Immunol. 2003;4:540–5.
29. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A. 2006;103:12481–6.
30. Hirao H, Jinnin M, Ichihara A, Fujisawa A, Makino K, Kajihara I, Sakai K, Fukushima S, Inoue Y, Ihn H. Detection of hair root miR-19a as a novel diagnostic marker for psoriasis. Eur J Dermatol. 2013;23:807–11.
31. Zibert JR, Lovendorf MB, Litman T, Olsen J, Kaczkowski B, Skov L. MicroRNAs and potential target interactions in psoriasis. J Dermatol Sci. 2010;58:177–85.
32. Xu N, Meisgen F, Butler LM, Han G, Wang XJ, Soderberg-Naucler C, Stahle M, Pivarcsi A, Sonkoly E. MicroRNA-31 is overexpressed in psoriasis and modulates inflammatory cytokine and chemokine production in keratinocytes via targeting serine/threonine kinase 40. J Immunol. 2013;190:678–88.
33. Lerman G, Avivi C, Mardoukh C, Barzilai A, Tessone A, Gradus B, Pavlotsky F, Barshack I, Polak-Charcon S, Orenstein A, Hornstein E, Sidi Y, Avni D. MiRNA expression in psoriatic skin: reciprocal regulation of hsa-miR-99a and IGF-1R. PLoS One. 2011;6, e20916.
34. Lerman G, Sharon M, Leibowitz-Amit R, Sidi Y, Avni D. The crosstalk between IL-22 signaling and miR-197 in human keratinocytes. PLoS One. 2014;9, e107467.
35. Ichihara A, Jinnin M, Yamane K, Fujisawa A, Sakai K, Masuguchi S, Fukushima S, Maruo K, Ihn H. microRNA-mediated keratinocyte hyperproliferation in psoriasis vulgaris. Br J Dermatol. 2011;165:1003–10.
36. Dai Y, Huang YS, Tang M, Lv TY, Hu CX, Tan YH, Xu ZM, Yin YB. Microarray analysis of microRNA expression in peripheral blood cells of systemic lupus erythematosus patients. Lupus. 2007;16:939–46.
37. Ding S, Liang Y, Zhao M, Liang G, Long H, Zhao S, Wang Y, Yin H, Zhang P, Zhang Q, Lu Q. Decreased microRNA-142-3p/5p expression causes CD4+ T cell activation and B cell hyperstimulation in systemic lupus erythematosus. Arthritis Rheum. 2012;64:2953–63.
38. Tang Y, Luo X, Cui H, Ni X, Yuan M, Guo Y, Huang X, Zhou H, de Vries N, Tak PP, Chen S, Shen N. MicroRNA-146a contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. Arthritis Rheum. 2009;60:1065–75.
39. Lu MC, Lai NS, Chen HC, Yu HC, Huang KY, Tung CH, Huang HB, Yu CL. Decreased microRNA(miR)-145 and increased miR-224 expression in T cells from patients with systemic lupus erythematosus involved in lupus immunopathogenesis. Clin Exp Immunol. 2013;171:91–9.
40. Fan W, Liang D, Tang Y, Qu B, Cui H, Luo X, Huang X, Chen S, Higgs BW, Jallal B, Yao Y, Harley JB, Shen N. Identification of microRNA-31 as a novel regulator contributing to impaired interleukin-2 production in T cells from patients with systemic lupus erythematosus. Arthritis Rheum. 2012;64:3715–25.
41. Te JL, Dozmorov IM, Guthridge JM, Nguyen KL, Cavett JW, Kelly JA, Bruner GR, Harley JB, Ojwang JO. Identification of unique microRNA signature associated with lupus nephritis. PLoS One. 2010;5, e10344.
42. Liu Y, Dong J, Mu R, Gao Y, Tan X, Li Y, Li Z, Yang G. MicroRNA-30a promotes B cell hyperactivity in patients with systemic lupus erythematosus by direct interaction with Lyn. Arthritis Rheum. 2013;65:1603–11.
43. Fu L, Jin L, Yan L, Shi J, Wang H, Zhou B, Wu X: Comprehensive review of genetic association studies and meta-analysis on mirna polymorphisms and rheumatoid arthritis and systemic lupus erythematosus susceptibility. Hum Immunol. 2014.
44. Koba S, Jinnin M, Inoue K, Nakayama W, Honda N, Makino K, Kajihara I, Makino T, Fukushima S, Ihn H. Expression analysis of multiple microRNAs in each patient with scleroderma. Exp Dermatol. 2013;22:489–91.

45. Takemoto R, Jinnin M, Wang Z, Kudo H, Inoue K, Nakayama W, Ichihara A, Igata T, Kajihara I, Fukushima S, Ihn H. Hair miR-29a levels are decreased in patients with scleroderma. Exp Dermatol. 2013;22:832–3.
46. Maurer B, Stanczyk J, Jungel A, Akhmetshina A, Trenkmann M, Brock M, Kowal-Bielecka O, Gay RE, Michel BA, Distler JH, Gay S, Distler O. MicroRNA-29, a key regulator of collagen expression in systemic sclerosis. Arthritis Rheum. 2010;62:1733–43.
47. Wang Z, Jinnin M, Kudo H, Inoue K, Nakayama W, Honda N, Makino K, Kajihara I, Fukushima S, Inoue Y, Ihn H. Detection of hair-microRNAs as the novel potent biomarker: evaluation of the usefulness for the diagnosis of scleroderma. J Dermatol Sci. 2013;72:134–41.
48. Sing T, Jinnin M, Yamane K, Honda N, Makino K, Kajihara I, Makino T, Sakai K, Masuguchi S, Fukushima S, Ihn H. MicroRNA-92a expression in the sera and dermal fibroblasts increases in patients with scleroderma. Rheumatology (Oxford). 2012;51:1550–6.
49. Shimada S, Jinnin M, Ogata A, Makino T, Kajihara I, Makino K, Honda N, Nakayama W, Inoue K, Fukushima S, Ihn H. Serum miR-21 levels in patients with dermatomyositis. Clin Exp Rheumatol. 2013;31:161–2.
50. Koga Y, Jinnin M, Ichihara A, Fujisawa A, Moriya C, Sakai K, Fukushima S, Inoue Y, Ihn H. Analysis of expression pattern of serum microRNA levels in patients with psoriasis. J Dermatol Sci. 2014;74:170–1.
51. Honda N, Jinnin M, Kira-Etoh T, Makino K, Kajihara I, Makino T, Fukushima S, Inoue Y, Okamoto Y, Hasegawa M, Fujimoto M, Ihn H. MiR-150 down-regulation contributes to the constitutive type I collagen overexpression in scleroderma dermal fibroblasts via the induction of integrin beta3. Am J Pathol. 2013;182:206–16.
52. Oshikawa Y, Jinnin M, Makino T, Kajihara I, Makino K, Honda N, Nakayama W, Inoue K, Fukushima S, Ihn H. Decreased miR-7 expression in the skin and sera of patients with dermatomyositis. Acta Derm Venereol. 2013;93:273–6.
53. Kawashita Y, Jinnin M, Makino T, Kajihara I, Makino K, Honda N, Masuguchi S, Fukushima S, Inoue Y, Ihn H. Circulating miR-29a levels in patients with scleroderma spectrum disorder. J Dermatol Sci. 2011;61:67–9.
54. Lovendorf MB, Zibert JR, Gyldenlove M, Ropke MA, Skov L. MicroRNA-223 and miR-143 are important systemic biomarkers for disease activity in psoriasis. J Dermatol Sci. 2014;75:133–9.
55. Honda N, Jinnin M, Kajihara I, Makino T, Makino K, Masuguchi S, Fukushima S, Okamoto Y, Hasegawa M, Fujimoto M, Ihn H. TGF-beta-mediated downregulation of microRNA-196a contributes to the constitutive upregulated type I collagen expression in scleroderma dermal fibroblasts. J Immunol. 2012;188:3323–31.
56. Inoue K, Jinnin M, Yamane K, Makino T, Kajihara I, Makino K, Honda N, Nakayama W, Fukushima S, Ihn H. Down-regulation of miR-223 contributes to the formation of Gottron's papules in dermatomyositis via the induction of PKCvarepsilon. Eur J Dermatol. 2013;23:160–7.
57. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55:74–108.
58. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. Nature. 2005;435:834–8.
59. Blower PE, Verducci JS, Lin S, Zhou J, Chung JH, Dai Z, Liu CG, Reinhold W, Lorenzi PL, Kaldjian EP, Croce CM, Weinstein JN, Sadee W. MicroRNA expression profiles for the NCI-60 cancer cell panel. Mol Cancer Ther. 2007;6:1483–91.
60. Gaur A, Jewell DA, Liang Y, Ridzon D, Moore JH, Chen C, Ambros VR, Israel MA. Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. Cancer Res. 2007;67:2456–68.
61. Mueller DW, Rehli M, Bosserhoff AK. MiRNA expression profiling in melanocytes and melanoma cell lines reveals miRNAs associated with formation and progression of malignant melanoma. J Invest Dermatol. 2009;129:1740–51.

62. Satzger I, Mattern A, Kuettler U, Weinspach D, Voelker B, Kapp A, Gutzmer R. MicroRNA-15b represents an independent prognostic parameter and is correlated with tumor cell proliferation and apoptosis in malignant melanoma. Int J Cancer. 2010;126:2553–62.
63. Philippidou D, Schmitt M, Moser D, Margue C, Nazarov PV, Muller A, Vallar L, Nashan D, Behrmann I, Kreis S. Signatures of microRNAs and selected microRNA target genes in human melanoma. Cancer Res. 2010;70:4163–73.
64. Sand M, Skrygan M, Sand D, Georgas D, Gambichler T, Hahn SA, Altmeyer P, Bechara FG. Comparative microarray analysis of microRNA expression profiles in primary cutaneous malignant melanoma, cutaneous malignant melanoma metastases, and benign melanocytic nevi. Cell Tissue Res. 2013;351:85–98.
65. Molnar V, Tamasi V, Bakos B, Wiener Z, Falus A. Changes in miRNA expression in solid tumors: an miRNA profiling in melanomas. Semin Cancer Biol. 2008;18:111–22.
66. Zhang Z, Sun H, Dai H, Walsh RM, Imakura M, Schelter J, Burchard J, Dai X, Chang AN, Diaz RL, Marszalek JR, Bartz SR, Carleton M, Cleary MA, Linsley PS, Grandori C. MicroRNA miR-210 modulates cellular response to hypoxia through the MYC antagonist MNT. Cell Cycle. 2009;8:2756–68.
67. Levati L, Alvino E, Pagani E, Arcelli D, Caporaso P, Bondanza S, Di Leva G, Ferracin M, Volinia S, Bonmassar E, Croce CM, D'Atri S. Altered expression of selected microRNAs in melanoma: antiproliferative and proapoptotic activity of miRNA-155. Int J Oncol. 2009;35:393–400.
68. Schultz J, Lorenz P, Gross G, Ibrahim S, Kunz M. MicroRNA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth. Cell Res. 2008;18:549–57.
69. Kozubek J, Ma Z, Fleming E, Duggan T, Wu R, Shin DG, Dadras SS. In-depth characterization of microRNA transcriptome in melanoma. PLoS One. 2013;8, e72699.
70. Caramuta S, Egyhazi S, Rodolfo M, Witten D, Hansson J, Larsson C, Lui WO. MicroRNA expression profiles associated with mutational status and survival in malignant melanoma. J Invest Dermatol. 2010;130:2062–70.
71. Greenberg E, Hershkovitz L, Itzhaki O, Hajdu S, Nemlich Y, Ortenberg R, Gefen N, Edry L, Modai S, Keisari Y, Besser MJ, Schachter J, Shomron N, Markel G. Regulation of cancer aggressive features in melanoma cells by microRNAs. PLoS One. 2011;6, e18936.
72. Penna E, Orso F, Cimino D, Tenaglia E, Lembo A, Quaglino E, Poliseno L, Haimovic A, Osella-Abate S, De Pitta C, Pinatel E, Stadler MB, Provero P, Bernengo MG, Osman I, Taverna D. MicroRNA-214 contributes to melanoma tumour progression through suppression of TFAP2C. EMBO J. 2011;30:1990–2007.
73. Levy C, Khaled M, Iliopoulos D, Janas MM, Schubert S, Pinner S, Chen PH, Li S, Fletcher AL, Yokoyama S, Scott KL, Garraway LA, Song JS, Granter SR, Turley SJ, Fisher DE, Novina CD. Intronic miR-211 assumes the tumor suppressive function of its host gene in melanoma. Mol Cell. 2010;40:841–9.
74. Mazar J, DeYoung K, Khaitan D, Meister E, Almodovar A, Goydos J, Ray A, Perera RJ. The regulation of miRNA-211 expression and its role in melanoma cell invasiveness. PLoS One. 2010;5, e13779.
75. Chan E, Patel R, Nallur S, Ratner E, Bacchiocchi A, Hoyt K, Szpakowski S, Godshalk S, Ariyan S, Sznol M, Halaban R, Krauthammer M, Tuck D, Slack FJ, Weidhaas JB. MicroRNA signatures differentiate melanoma subtypes. Cell Cycle. 2011;10:1845–52.
76. Glud M, Rossing M, Hother C, Holst L, Hastrup N, Nielsen FC, Gniadecki R, Drzewiecki KT. Downregulation of miR-125b in metastatic cutaneous malignant melanoma. Melanoma Res. 2010;20:479–84.
77. Xu Y, Brenn T, Brown ER, Doherty V, Melton DW. Differential expression of microRNAs during melanoma progression: miR-200c, miR-205 and miR-211 are downregulated in melanoma and act as tumour suppressors. Br J Cancer. 2012;106:553–61.
78. Poliseno L, Haimovic A, Segura MF, Hanniford D, Christos PJ, Darvishian F, Wang J, Shapiro RL, Pavlick AC, Berman RS, Hernando E, Zavadil J, Osman I. Histology-specific microRNA alterations in melanoma. J Invest Dermatol. 2012;132:1860–8.

79. Segura MF, Belitskaya-Levy I, Rose AE, Zakrzewski J, Gaziel A, Hanniford D, Darvishian F, Berman RS, Shapiro RL, Pavlick AC, Osman I, Hernando E. Melanoma microRNA signature predicts post-recurrence survival. Clin Cancer Res. 2010;16:1577–86.
80. Stark MS, Tyagi S, Nancarrow DJ, Boyle GM, Cook AL, Whiteman DC, Parsons PG, Schmidt C, Sturm RA, Hayward NK. Characterization of the melanoma miRNAome by deep sequencing. PLoS One. 2010;5, e9685.
81. Hanna JA, Hahn L, Agarwal S, Rimm DL. In situ measurement of miR-205 in malignant melanoma tissue supports its role as a tumor suppressor microRNA. Lab Invest. 2012;92:1390–7.
82. Kunz M. MicroRNAs in melanoma biology. Adv Exp Med Biol. 2013;774:103–20.
83. Esquela-Kerscher A, Slack FJ. OncomiRs—microRNAs with a role in cancer. Nat Rev Cancer. 2006;6:259–69.
84. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ. RAS is regulated by the let-7 microRNA family. Cell. 2005;120:635–47.
85. Wolfel T, Hauer M, Schneider J, Serrano M, Wolfel C, Klehmann-Hieb E, De Plaen E, Hankeln T, Meyer zum Buschenfelde KH, Beach D. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science. 1995;269:1281–4.
86. Chudnovsky Y, Khavari PA, Adams AE. Melanoma genetics and the development of rational therapeutics. J Clin Invest. 2005;115:813–24.
87. Miller AJ, Mihm Jr MC. Melanoma. N Engl J Med. 2006;355:51–65.
88. Couts KL, Anderson EM, Gross MM, Sullivan K, Ahn NG. Oncogenic B-Raf signaling in melanoma cells controls a network of microRNAs with combinatorial functions. Oncogene. 2013;32:1959–70.
89. Fu X, Meng Z, Liang W, Tian Y, Wang X, Han W, Lou G, Lou F, Yen Y, Yu H, Jove R, Huang W. miR-26a enhances miRNA biogenesis by targeting Lin28B and Zcchc11 to suppress tumor growth and metastasis. Oncogene. 2014;33:4296–306.
90. Cai Q, Sun MH, Fu G, Ding CW, Mo SJ, Cai SJ, Ren SX, Min DL, Xu XL, Zhu WP, Zhang TM, Shi DR. [Mutation analysis of hMSH2 and hMLH1 genes in Chinese hereditary nonpolyposis colorectal cancer families]. Zhonghua Bing Li Xue Za Zhi. 2003;32:323–8.
91. Liu S, Tetzlaff MT, Cui R, Xu X. miR-200c inhibits melanoma progression and drug resistance through down-regulation of BMI-1. Am J Pathol. 2012;181:1823–35.
92. Hurteau GJ, Carlson JA, Spivack SD, Brock GJ. Overexpression of the microRNA hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin. Cancer Res. 2007;67:7972–6.
93. Montone KT, van Belle P, Elenitsas R, Elder DE. Proto-oncogene c-kit expression in malignant melanoma: protein loss with tumor progression. Mod Pathol. 1997;10:939–44.
94. Shen SS, Zhang PS, Eton O, Prieto VG. Analysis of protein tyrosine kinase expression in melanocytic lesions by tissue array. J Cutan Pathol. 2003;30:539–47.
95. He H, Jazdzewski K, Li W, Liyanarachchi S, Nagy R, Volinia S, Calin GA, Liu CG, Franssila K, Suster S, Kloos RT, Croce CM, de la Chapelle A. The role of microRNA genes in papillary thyroid carcinoma. Proc Natl Acad Sci U S A. 2005;102:19075–80.
96. Felli N, Fontana L, Pelosi E, Botta R, Bonci D, Facchiano F, Liuzzi F, Lulli V, Morsilli O, Santoro S, Valtieri M, Calin GA, Liu CG, Sorrentino A, Croce CM, Peschle C. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. Proc Natl Acad Sci U S A. 2005;102:18081–6.
97. Felicetti F, Errico MC, Bottero L, Segnalini P, Stoppacciaro A, Biffoni M, Felli N, Mattia G, Petrini M, Colombo MP, Peschle C, Care A. The promyelocytic leukemia zinc finger-microRNA-221/-222 pathway controls melanoma progression through multiple oncogenic mechanisms. Cancer Res. 2008;68:2745–54.
98. Garofalo M, Di Leva G, Romano G, Nuovo G, Suh SS, Ngankeu A, Taccioli C, Pichiorri F, Alder H, Secchiero P, Gasparini P, Gonelli A, Costinean S, Acunzo M, Condorelli G, Croce CM. miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. Cancer Cell. 2009;16:498–509.

99. Errico MC, Felicetti F, Bottero L, Mattia G, Boe A, Felli N, Petrini M, Bellenghi M, Pandha HS, Calvaruso M, Tripodo C, Colombo MP, Morgan R, Care A. The abrogation of the HOXB7/PBX2 complex induces apoptosis in melanoma through the miR-221&222-c-FOS pathway. Int J Cancer. 2013;133:879–92.
100. Satzger I, Mattern A, Kuettler U, Weinspach D, Niebuhr M, Kapp A, Gutzmer R. MicroRNA-21 is upregulated in malignant melanoma and influences apoptosis of melanocytic cells. Exp Dermatol. 2012;21:509–14.
101. Talotta F, Cimmino A, Matarazzo MR, Casalino L, De Vita G, D'Esposito M, Di Lauro R, Verde P. An autoregulatory loop mediated by miR-21 and PDCD4 controls the AP-1 activity in RAS transformation. Oncogene. 2009;28:73–84.
102. Segura MF, Hanniford D, Menendez S, Reavie L, Zou X, Alvarez-Diaz S, Zakrzewski J, Blochin E, Rose A, Bogunovic D, Polsky D, Wei J, Lee P, Belitskaya-Levy I, Bhardwaj N, Osman I, Hernando E. Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor. Proc Natl Acad Sci U S A. 2009;106:1814–9.
103. Segura MF, Greenwald HS, Hanniford D, Osman I, Hernando E. MicroRNA and cutaneous melanoma: from discovery to prognosis and therapy. Carcinogenesis. 2012;33:1823–32.
104. Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, Benjamin H, Kushnir M, Cholakh H, Melamed N, Bentwich Z, Hod M, Goren Y, Chajut A. Serum microRNAs are promising novel biomarkers. PLoS One. 2008;3, e3148.
105. Leidinger P, Keller A, Borries A, Reichrath J, Rass K, Jager SU, Lenhof HP, Meese E. High-throughput miRNA profiling of human melanoma blood samples. BMC Cancer. 2010;10:262.
106. Nguyen T, Kuo C, Nicholl MB, Sim MS, Turner RR, Morton DL, Hoon DS. Downregulation of microRNA-29c is associated with hypermethylation of tumor-related genes and disease outcome in cutaneous melanoma. Epigenetics. 2011;6:388–94.
107. Friedman EB, Shang S, de Miera EV, Fog JU, Teilum MW, Ma MW, Berman RS, Shapiro RL, Pavlick AC, Hernando E, Baker A, Shao Y, Osman I. Serum microRNAs as biomarkers for recurrence in melanoma. J Transl Med. 2012;10:155.
108. Ratushny V, Gober MD, Hick R, Ridky TW, Seykora JT. From keratinocyte to cancer: the pathogenesis and modeling of cutaneous squamous cell carcinoma. J Clin Invest. 2012;122:464–72.
109. Jarus-Dziedzic K, Zub W, Dziedzic D, Jelen M, Krotochwil J, Mierzejewski M. Multiple metastases of carcinoma basocellulare into spinal column. J Neurooncol. 2000;48:57–62.
110. Heffelfinger C, Ouyang Z, Engberg A, Leffell DJ, Hanlon AM, Gordon PB, Zheng W, Zhao H, Snyder MP, Bale AE. Correlation of global microRNA expression with basal cell carcinoma subtype. G3 (Bethesda). 2012;2:279–86.
111. Sand M, Skrygan M, Sand D, Georgas D, Hahn SA, Gambichler T, Altmeyer P, Bechara FG. Expression of microRNAs in basal cell carcinoma. Br J Dermatol. 2012;167:847–55.
112. Cassarino DS, Derienzo DP, Barr RJ. Cutaneous squamous cell carcinoma: a comprehensive clinicopathologic classification. Part one. J Cutan Pathol. 2006;33:191–206.
113. Kwa RE, Campana K, Moy RL. Biology of cutaneous squamous cell carcinoma. J Am Acad Dermatol. 1992;26:1–26.
114. Sand M, Skrygan M, Georgas D, Sand D, Hahn SA, Gambichler T, Altmeyer P, Bechara FG. Microarray analysis of microRNA expression in cutaneous squamous cell carcinoma. J Dermatol Sci. 2012;68:119–26.

Chapter 17
microRNA and Allergy

Ana Rebane

Abstract Allergy is a common hypersensitivity disorder of the immune system, which, along with other factors, is also subjected to regulation by microRNAs. The most common allergic diseases are allergic rhinitis, asthma, atopic dermatitis, and food allergy, which all are multifactorial and very heterogeneous conditions, highlighting the need for more individualized treatment techniques. More particular key questions in relation to allergic diseases are how microRNAs influence the differentiation, polarization, plasticity and functions of T helper and other immune cells, as well as the development of immune tolerance. In addition, microRNAs can affect allergic inflammation and tissue remodeling through their functions in epithelial and other tissue cells. Among immune system-related microRNAs, miR-21, miR-146a, and miR-155 are the most intensively studied and have convincingly been demonstrated to regulate immune responses and tissue inflammation in allergic diseases. Further characterization of microRNA functions is important, as similar to other conditions, the modulation of microRNA expression could potentially be used for therapeutic purposes in allergic diseases in the future. In addition, miRNAs could be implemented as biomarkers for endotyping complex allergic conditions.

Keywords Noncoding RNA • Allergy • Asthma • Rhinitis • Dermatitis • Food allergy

Introduction

Immune responses in health and disease are controlled by the dynamic and multifactorial regulation of gene expression in each cell type involved. A healthy immune system detects and eliminates pathogens very efficiently. However, excessive and inappropriate responses to self-antigens, pathogens, tissue damage, toxins, irritants, and allergens may cause unreasonably strong inflammation, leading to autoimmune

A. Rebane (✉)
Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia
e-mail: ana.rebane@ut.ee

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_17

or allergic diseases [1–3]. The most common allergic diseases are allergic rhinitis, asthma, and atopic dermatitis (AD), which can occur either alone or in combination [4, 5]. A predisposition toward developing certain allergic reactions is also known as atopy. Immunologically, atopy is characterized by the expansion of the Th2 cell subset and IgE-expressing memory/effector B cells and plasma cells producing IgE antibodies specific for common environmental allergens. Because allergic diseases mainly affect tissues that interface with the environment, the role of epithelial cell responses is important. In addition, various nonpathogenic and pathogenic microbes and viruses affecting the same tissues enhance the complexity of the molecular events that lead to or protect against the development of allergic inflammation [6]. For instance, human rhinoviruses (HRVs), which typically only cause the common cold, may also cause long-lasting infection and acute asthma exacerbations in asthmatics [7, 8].

Atopic diseases possess a strong genetic component, which is well characterized for AD. Numerous genetic studies of AD have identified candidate genes linked either to the epidermal barrier, such as filaggrin (*FLG*) [9], or to immune processes, such as *IL4*/*IL13* receptor [10, 11], *IL13* [12] and the α-chain of the high affinity receptor for IgE, *FCER1A* [13–15]. Distinct functional *IL13* variants have been associated with asthma susceptibility as well [16]. A recent meta-analysis of genome-wide association studies confirms previous findings regarding the involvement of both the epidermal barrier function and immune dysregulation in AD pathogenesis [17].

Many studies have addressed the domination of Th2 cells in atopic diseases. As one possibility, high IFN-γ-secreting Th1 cells in peripheral blood selectively undergo activation-induced cell death and skew the immune response toward surviving Th2 cells in AD patients [18]. Albeit Th2 cells are dominant in the periphery of atopic patients, Th1 type responses, such as an enhanced level of IFN-γ, in fact appear to have a stronger influence on epithelial cells in the chronic phase of inflammation [14, 19–23].

Allergic diseases are very heterogeneous [24]. For example, both asthma and AD were initially divided into either extrinsic or allergic or intrinsic, i.e., nonatopic, disease. Recently, it has become clear that asthma and AD are far more complex diseases composed of a number of variants with different underlying pathophysiologies. Accordingly, it has been proposed that asthma can be divided into distinct disease entities called asthma endotypes, which link both the clinical characteristics and underlying biology [25]. However, the currently used diagnostic techniques and biomarkers do not allow for precise asthma endotyping.

The existing therapies for atopic diseases aim to control the symptoms. Despite extensive research, there is no established method to prevent asthma and AD, and the severe forms of these diseases are not well controlled [3]. Therefore, further characterization of the molecular mechanisms contributing to the pathogenesis of allergic diseases is important and ultimately could help in the development of more personalized therapeutic approaches for these diseases.

Although studies on the functions of microRNAs in allergic diseases have emerged during the last 5 years, the roles of miRNAs in the regulation and pathogenesis of these diseases remain largely uncharacterized [26, 27]. Here, we provide an overview of the microRNAs that influence the development of allergic

diseases or impact the strength of inflammation in affected tissues. In addition, we discuss the potential of microRNAs to serve as biomarkers and targets of gene therapy in allergic diseases.

Three Guardians of the Immune System

Three microRNAs, miR-21, miR-146a, and miR-155, are most likely the most intensively studied immune system-related miRNAs and have also been shown to play important roles in the development of allergic diseases and inflammation in several studies. The functions and best characterized targets of miR-21, miR-146a, and miR-155 are presented in Fig. 17.1.

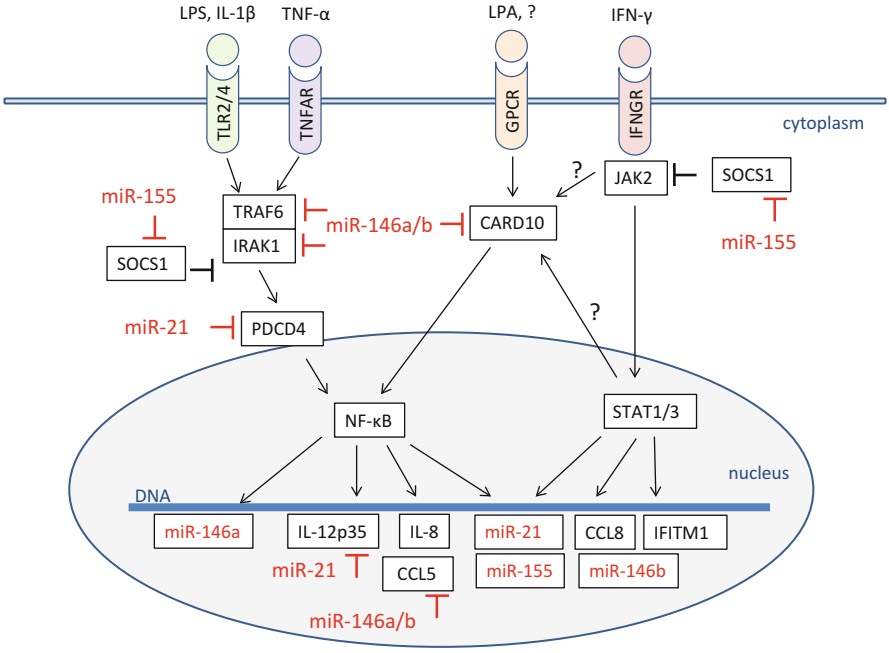

Fig. 17.1 The expression regulation and functions of miR-146a/b, miR-21, and miR-155. The expression of miR-146a and miR-146b can be stimulated by activation of the NF-κB and JAK-STAT signaling, respectively, while the expression of miR-21 and miR-155 can be upregulated by both these pathways. miR-21 inhibits inflammatory responses by targeting tumor suppressors and a pro-inflammatory protein (PDCD4) that promotes the activation of [30] and contributes to Th2 polarization by targeting IL-12p35 [53]. miR-146a/b inhibit most probably the same set of target genes. miR-146a/b directly downregulate IL-1 receptor-associated kinase 1 (IRAK1), TNF receptor-associated factor 6 (TRAF6) [31], which can be activated by TLR ligands, TNF-α and IL-1β, as well as caspase recruitment domain-containing protein 10 (CARD10), which can be activated through G protein-coupled receptors (GPCRs) by lysophosphatidic acid (LPA) or other unidentified factors, as well as by IFN-γ in unknown manner [33, 34]. miR-155 promotes type I IFN signaling and contributes to the activation of NF-κB pathway by targeting suppressor of cytokine signaling 1, SOCS1 [41]

miR-21 is mainly studied in connection with cancer, but it has been shown to regulate several immunological processes as well [28]. miR-21 is highly expressed in many cell types and can be further upregulated by signal transducers and activators of transcription 3 (STAT3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [29]. In mouse macrophages, miR-21 has been shown to inhibit inflammatory responses by targeting tumor suppressors and a pro-inflammatory protein (PDCD4) that promotes the activation of NF-κB and controls the production of immune-suppressor cytokine IL-10 [30].

Similarly, miR-146a is an anti-inflammatory miRNA known to inhibit the activation of the NF-κB pathway. miR-146a directly downregulates IL-1 receptor-associated kinase 1 (IRAK1), TNF receptor-associated factor 6 (TRAF6) [31], Relb, a member of the non-canonical NF-κB/Rel family [32], and caspase recruitment domain-containing protein 10 (CARD10, also known as CARD recruited membrane-associated protein 3, CARMA3) [33, 34]. miR-146a−/− mice develop autoimmunity at approximately 6 months of age, which is caused by the overactivation of STAT1 in Treg cells, leading to increased IFN-γ production and an inability to suppress immune responses mediated by Th1 cells [35–37]. Recent studies demonstrate that in addition to miR-146a, miR-146b is expressed and plays important roles in several different cell types [38, 39]. Mature miR-146a and miR-146b differ from each other by two nucleotides, and they target the same or a very similar set of genes because of their identical seed sequence [31, 40]. Although the function of miR-146a has been studied intensively in immune system and in numerous diseases, the function and regulation of miR-146b is less well described. In monocytes, miR-146b is upregulated via an IL-10-mediated STAT3-dependent loop upon LPS stimulation and has been shown to directly target TLR4 [38]. In normal breast epithelial cells, the expression of miR-146b is induced by IL-6 and IFN-γ through activation of the STAT3 and STAT1 pathways, respectively [39].

In contrast to miR-21 and miR-146a/b, miR-155 acts primarily as a pro-inflammatory factor. miR-155 expression is induced upon vesicular stomatitis virus infection in macrophages, which promotes type I IFN signaling by targeting suppressor of cytokine signaling 1, SOCS1 [41]. The suppression of SH2 domain-containing inositol-5-phosphatase (SHIP1) by miR-155 results in activation of Akt kinase and the upregulation of IFN response genes during the cellular response to LPS [42]. In addition, miR-155 directly represses BCL6, a transcription factor that attenuates NF-κB signaling, and this promotes atherosclerosis in mice [43]. miR-155 also functions in the development and activation of adaptive immune cells, including effector T cell subsets [44]. In addition, miR-155-deficient mice are defective in production of Th1 and Th17 cells and, therefore, are highly resistant to experimental autoimmune encephalomyelitis [45].

Numerous other miRNAs control innate and adaptive immune responses. For example, miR-301a, miR-9, miR-147b, and miR-125a have been shown to influence the activation of the NF-κB pathway [46], and miR-10a, miR-17-92, miR-181a, miR-182, and miR-29a/b impact the differentiation and plasticity of T cells [47]. In addition, miR-17-92, miR-34a, miR-150, miR-181b, miR-125b, and miR-217 affect the development and functions of B cells [48]. Within the context of

allergic diseases, it is particularly interesting to study how miRNAs influence Th2 polarization, Treg cell suppression capacity, and Ig class switching in B cells.

miRNAs in the Regulation of Effector T Cell Responses in Allergic Diseases

Naïve T cells can differentiate into Th1, Th2, or Th17 cells, all of which can promote different types of inflammatory responses. Moreover, the established subsets of effector T cells are rather heterogeneous cell populations, which often express only one or two of the lineage specific cytokines and under certain conditions can coproduce different amounts of the cytokines characteristic of other lineages, thus leading to different functional outcomes [49–51]. Allergic diseases are characterized by the expansion of Th2 cells and IgE-expressing memory B cells and plasma cells that produce IgE antibodies specific to common environmental allergens [3].

The characteristic cytokines for Th2 cells are IL-5, IL-4, and IL-13; however, the Th1/Th2 balance is also influenced by Th1 cytokines, such as IFN-γ and IL-12, and by innate immune responses of antigen presenting cells (APCs), dendritic cells (DCs), and tissue macrophages, and responses of epithelial cells [52]. For example, epithelial cells produce thymic stromal lymphopoietin (TSLP) in response to various stimuli and that contributes to activation of Th2 type responses. Coordinately, all miRNAs regulating the balance of Th1/Th2 type cytokines and innate immune responses in APCs and epithelial cells influence the pathogenesis of allergic diseases. For example, in a mouse model of ovalbumin (OVA)-induced allergic airway inflammation, miR-21 was the most highly induced miRNA in transgenic mice overexpressing IL-13 in the lungs. Because miR-21 directly targets IL-12p35, it was suggested that miR-21 might contribute to Th2 polarization [53]. Consistent with this, OVA-challenged miR-21-deficient mice exhibited reduced lung eosinophilia and increased levels of IFN-γ when compared to wild-type mice [54]. miRNA-21 also has been shown to inhibit innate immune responses in TLR2 agonist-induced lung inflammation in mice [55] (Table 17.1).

In line with research in mouse models of airway inflammation, a correlation between reduced levels of miR-21 in mononuclear leukocytes from human umbilical cord blood samples and elevated antenatal IgE production in allergic rhinitis patients have been observed [56]. Additionally, the upregulation of miR-21 was observed in esophageal biopsies from patients with eosinophilic esophagitis (EoE) [57] (Table 17.2). EoE is characterized by very high IL-13 overproduction, very strong Th2 type inflammation and eosinophilia in the esophagus and can therefore regarded as a model for Th2-type diseases [58]. miR-21 is also upregulated in lesional skin from subjects with allergic responses to diphenylcyclopropenone (DPCP) in humans and in a dinitrofluorobenzene (DNFB)-induced contact dermatitis mouse model, conditions in which Th17 and Th1 responses are dominant [59].

Ubiquitously expressed miRNA let-7 has been shown to influence the expression of IL-13 in lung epithelial cell-line A549. Indeed, intranasal administration of let-7

Table 17.1 The functions of miRNAs in asthma

miRNA	Function	Targets
miR-126	Inhibition reduced allergic inflammation in an HDM-induced mouse model of asthma [78] and eosinophilia in a mouse model of chronic airway inflammation [79]	?
miR-221	Upregulated in OVA-induced mouse models of asthma; silencing reduced allergic inflammation [82]	Spred-2
miR-145	Silencing reduced allergic inflammation in an HDM-induced mouse model of asthma [80]	?
miR-106a	Silencing reduced allergic inflammation in an HDM-induced mouse model of asthma [81]	IL-10
let-7a	Overexpression reduced allergic inflammation in an OVA-induced mouse model of asthma [60]	IL-13
	Intravenous administration of LNA inhibitors impeded AHR in a mouse model of asthma [61]	
miR-21	Overexpressed in lung-specific IL-13-expressing transgenic mice with induced allergic airway inflammation [53]	IL-12p35
	Overexpression reduced TLR2 agonist-induced lung inflammation in mice [55]	
	Increased Th1 cytokines and reduced eosinophilia in an OVA-induced mouse model of asthma in miR-21-deficient mice [54]	
miR-146a	Overexpressed in splenic CD4+ T lymphocytes [76]	IRAK1, TRAF6, CARD10, indirectly inhibits COX-2 and IL-1β
	Reduced expression in CD8+ and CD4+ cells in patients under oral corticosteroid treatment [119]	
	Reduced cytokine induced apoptosis in human bronchial epithelial cells [94]	
	Increased in human asthmatic SMCs [93]	
miR-375	A positive regulator of TSLP in human primary lung epithelial cells [65]	?
miR-133a	Downregulated in mouse bronchial smooth muscle cells; augments airway contraction and hyperresponsiveness [89]	RhoA
miR-1	Downregulated by VEGF in lung endothelium; intranasal administration inhibited inflammatory responses to OVA, HDM, and IL-13 overexpression Mpl [88]	Mpl
miR-155	Required for Th2-mediated eosinophilic inflammation in an OVA-induced mouse model of asthma [77]	PU.1
	miR-155-deficient mice spontaneously developed asthma-like inflammation in the lung and showed increased levels of IL-4 and IL-5 in T cells [44]	c-Maf
	Increased in asthmatic SMCs upon stimulation [95]	
miR-17~92 cluster	Downregulated in pulmonary hypertension, SMCs; specific depletion augments hypoxia-induced pulmonary hypertension in mice [91]	PDLIM5
miR-708	Inhibits airway SMC contractility [92]	CD38

Table 17.2 The functions of miRNAs in allergic diseases other than asthma

miRNA	Disease	Function	Targets
miR-21	Allergic rhinitis, ACD, EoE	Antenatal IgE production and development of allergic rhinitis [56]	IL-12p35
		Upregulated in contact dermatitis lesional skin [59]	
		Upregulated in EoE esophageal tissue [57]	
		Inhibits Th1-associated delayed-type hypersensitivity cutaneous responses in miR-21-deficient mice [54]	
miR-146a	AD	Upregulated in the skin and keratinocytes of AD patients; alleviates chronic inflammation in a mouse model of AD [34]	IRAK1, CARD10, CCL5
miR-375	EoE	Expression is modulated by IL-13 [58]	?
		Downregulated in EoE esophageal tissue [57]	
miR-155	AD	Influences development of AD by downregulating CTLA-4 negative regulator of T cell activation [102]	CTLA-4
miR-125b	CRS with nasal polyps	Upregulated in CRS with nasal polyps; influences IFN-α/β production [103]	4E-BP1
miR-150	ACD	Nanovesicles containing miR-150 induce antigen-specific tolerance in a mouse ACD model [110]	

family miRNAs led to reduced IL-13 and AHR as well as the resolution of allergic inflammation in an OVA-induced mouse model of asthma [60]. However, in another study, instead of exacerbating the disease, let-7 miRNA inhibition using intravenously administered locked nucleic acid (LNA) inhibitors hampered AHR in a mouse model of asthma [61].

miR-155 can impact the Th1/Th2 balance and the development of allergic disease through its function in macrophages, in which miR-155 targets IL-13RA, SOCS1, and SHIP1. When miR-155 levels are reduced in differentiating macrophages, STAT6 becomes more activated, leading to the establishment of an alternative M2 pro-Th2 phenotype [62]. A very interesting recent study using miR-155(−/−) or miR-146a(−/−) T cells identified that T cell-intrinsic miR-155 is required for type-2 immunity and that T cell-intrinsic miR-146a is required to prevent overt Th1/Th17 skewing [63]. Thus, if there is no miR-155 in macrophages, they tend to stimulate Th2 responses; however, if there is no miR-155 in T cells, there is less differentiation toward Th2 type direction suggesting that tightly regulated miR-155 in each participating cell type is needed for proper functioning of the immune system.

miR-375 is yet another miRNA that contributes to the differentiation of Th2 cells. miR-375 has been shown to be induced by IL-13 in gut mucosal epithelial cells and it positively regulates the expression of key Th2 cytokines, TSLP, and resistin-like beta (RELMβ) [64]. In line with these results, diesel exhaust particles, which are known to aggravate asthma, induce TSLP mRNA and miR-375 in primary human

bronchial epithelial cells [65]. In contrast to gut mucosal epithelial cells, miR-375 is downregulated by IL-13 in human esophageal squamous and bronchial columnar epithelial cells, suggesting that the regulation of miR-375 is complicated and cell type specific [57].

In addition to the Th2 cells, recent studies propose an important role for Th22 cells in the chronic phase of the allergic tissue inflammation. Influence of miRNAs on development of Th22 cells, as well as on production of IL-22 has not been studied. This will be interesting because IL-22 might be also produced by Th17 cells; however, when IL-17 augments inflammation, IL-22 is known to play a tissue-protective role [66].

miRNAs and Regulatory T and B Cell Responses

T cells with immunoregulatory capacity can be classified into two groups: thymus-derivednaturally occurring CD4+CD25+FOXP3+ Treg cells and inducible Treg cells (iTreg) generated outside the thymus after antigenic stimulation. Importantly, the balance between allergen-specific Treg cells and Th2 cells is decisive in the development of allergy. Studies on the mechanisms of immune responses to allergens have demonstrated that iTreg cells are dominant in healthy individuals [67]. Therefore, miRNAs that influence the development and functions of iTregs most likely play important roles in allergic diseases. For example, miR-10a has been shown to limit the phenotypic conversion of iTreg cells into follicular helper T cells and hinder the differentiation of the Th17 cell subset [68]. An interesting large-scale overexpression screen with T cell-expressed miRNAs in naïve mouse $CD4^+$ T cells undergoing Treg differentiation identified 29 miRNAs with a negative effect and 10 miRNAs with a positive effect. miR-100, miR-99a, and miR-10b all specifically promoted Treg differentiation and inhibited the Th17 program without impacting viability, proliferation, and activation [69]. In addition to autoimmunity, Th17 cells play a dominant role in neutrophilic asthma, which is also associated with asthma exacerbations in response to rhinoviruses [70], indicating that these miRNAs potentially influence the development of neutrophilic asthma and asthma exacerbation.

iTreg cells not only suppress Th1 or Th2 cell responses but also have a direct influence on B cells. Both natural Tregs and iTregs reduce the IgE-secreting plasma cell frequency and simultaneously augment IgG4-secreting plasma cells. Recently, it has also been shown that regulatory IL-10-producing B cells induce Treg cells and alleviate symptoms in a murine model of allergic asthma [71, 72]. However, the roles of miRNAs in immunoregulatory B cells are almost fully unexplored. Interestingly, miR-21 was recently shown to stimulate B cells to produce IL-10 in a study exploring the role of miR-21 in nasopharyngeal cancer. Thus, in addition to its role in triggering Th2 type responses, miR-21 is required for the regulatory role of B cells, which suggests that similarly to miR-155, well-controlled miR-21 levels in every cell is needed for the proper functioning of the immune system.

The development of regulatory and effector T cells strongly depends on innate immune responses of APCs [73]. Several miRNAs may play important roles in the modulation of DC capacity to induce regulatory T cells. For example, miR-23b transfection into mouse or human in vitro differentiated DCs leads to increased IL-10 and decreased IL-12 production, inactivation of the Notch1 and NF-κB signaling pathways, and increased capacity of DCs to promote FoxP3+ CD4+ T cell proliferation [74]. Interestingly, miR-146a has been shown to be differentially expressed by myeloid DC subsets and it desensitizes DCs to TLR2-dependent activation, which suggests that high constitutive miR-146a levels in the epidermis render Langerhans cells (LCs) in the epithelium less susceptible to inappropriate activation by commensal bacterial through TLR2 [75]. Thus, both miR-146a and miR-23b might essentially contribute to protection against the development of allergic responses through their actinon in APCs, the first line of the cells activated by allergens. As the modulation of microRNA expression in APCs can be considered easier compared with T and B lymphocytes, further studies about the functions of microRNAs in APCs has high potential also in light of few of the development of therapeutic approaches for allergic diseases.

miRNAs in Asthma

The first study to demonstrate the importance of miRNAs in airway diseases was published in 2007 by Rodriguez et al., who characterized miR-155-deficient mice and noted that approximately 50 % of aging miR-155-deficient mice spontaneously develop asthma-like lung inflammation with increased Th2-type cytokines and greater numbers of lymphocytes and macrophages but similar counts of eosinophils compared to wild-type mice [44]. In ensuing studies, the expression of several miRNAs, including miR-155, was found to be dysregulated in the airways or lymphocytes of patients with asthma, as well as in different mouse models of airway inflammation [76]. Interestingly, the same miR-155-deficient mice were recently demonstrated as developing an altered inflammatory response with diminished eosinophilic inflammation, reduced eotaxin-2/CCL24 and periostin levels, and reduced Th2 cell numbers in an experimental mouse model of allergic airway inflammation [77]. Concordantly, miR-155 was shown to be required for Th2-type responses [63]. Thus, it appears that in the case of miR-155 deficiency, a spontaneous development of airway inflammation occurs; however, in induced mouse models, miR-155 rather contributes to airway inflammation.

In addition to miR-155, several other miRNAs have been shown to modulate asthma-like lung inflammation in different mouse models (Table 17.1). The expression of miR-126 was found to be increased in the airways of mice upon exposure to house dust mite (HDM), and the inhibition of miR-126 using intranasal administration of miR-126 antagomir reduced allergic inflammation in the same mouse model of asthma [78] and also decreased eosinophilia in a mouse model of chronic airway

inflammation [79]. Similarly, the inhibition of miR-145 [80] and miR-106a [81] had anti-inflammatory effects in HDM-induced allergic airways disease in mice. Similar to miR-126, enhanced expression of miRNA-221 was detected in the lung biopsies of mice subjected to an OVA-induced mouse model of asthma, and the inhibition of miR-221 reduced inflammation in the airways [82]. Interestingly, miR-221 is also known to regulate the cell cycle of mast cells and is upregulated by stimulation with IgE-allergen complexes in these cells [83, 84].

Current therapies for asthma aim to control symptoms and mainly include fast-acting beta2-adrenoceptor agonists and corticosteroids for long-term control, yet these therapies are inefficient in controlling severe forms of asthma and allergic diseases [3]. Thus, one important question in research concerning asthma is how miRNAs impact the development of corticosteroid-resistant and severe asthma. One of the initial studies in a mouse model of corticosteroid-resistant asthma demonstrated a rapid change in miRNA levels in mouse lungs following LPS-induced inflammation; however, no influence of glucocorticoids was detected [33]. Another study showed that the expression of miR-146a is reduced in CD8+ and CD4+ T cells from severe asthma patients under continuous oral corticosteroid treatment [34]. In agreement with this observation, the expression of miR-146a was shown to be suppressed by dexamethasone in splenic CD4+ T lymphocytes from mice subjected to an OVA-induced model of asthma [53].

Together, these studies show that miRNAs can either enhance or reduce airway inflammation and suggest that a complex network of miRNAs impacts immune responses in asthma. Because asthma is a complex syndrome with heterogeneous endotypes or phenotypes [25, 85], miRNAs as fine-tuners of gene expression also may contribute to the development of different forms of the disease.

miRNAs in Bronchial Epithelium and Airway Smooth Muscle Cells

Disease-related changes in lung alveolar epithelial cells, endothelial cells, fibroblasts, and bronchial smooth muscle cells (SMCs) have strong impact on the pathogenesis of asthma, and miRNAs are clearly part of the regulatory network involved in tissue responses to asthma. An initial study demonstrated that miRNAs have different expression profiles in airway SMCs, bronchial epithelial cell-lines A549 and Beas2B, bronchial fibroblasts, and alveolar macrophages [86]. In epithelial cells from asthmatic patients, 66 miRNAs were detected as differentially expressed, and pathway analysis indicated that the putative targets of these miRNAs encode disease- or inflammation-related proteins such as TNF-α, IL-8, Cox2, IL-6, and AQP4 [87].

Several recent studies describe the functions of miRNAs in lung endothelial cells and SMCs. The lung-specific overexpression of vascular endothelial growth factor (VEGF) was shown to decrease miR-1 expression in the lung endothelium,

whereas intranasally delivered miR-1 inhibited inflammatory responses to HDM, OVA, and IL-13. In the same study, VEGF blockade and the knockdown of a miR-1 target, myeloproliferative leukemia virus oncogene (MPL), inhibited Th2-mediated lung inflammation, suggesting that VEGF controls lung Th2 inflammation via miR-1 and MPL [88]. Suppression of miR-133a leads to increased expression of the small GTPase RhoA, causing augmented contraction and hyperresponsiveness of bronchial SMCs in a mouse model of allergic asthma [89]. miR-140-3p is another microRNA that possibly has a strong impact on SMCs, as it is capable of activating p38 MAPK and NF-κB in these cells [90]. Patients with pulmonary hypertension were found to express decreased levels of the miR-17~92 cluster, transforming growth factor beta (TGF-β), and SMC markers. Concordantly, SMC-specific knockout of miR-17-92 attenuated hypoxia-induced pulmonary hypertension (PH) in mice, suggesting a direct link between the downregulation of the miR-17-92 cluster and hypoxia-induced PH in these mice [91]. The cell-surface protein CD38, which mediates airway SMC contractility, was recently shown to be both directly and indirectly regulated by miR-708 through JNK MAPK and PI3K/AKT signaling pathways [92].

The expression of anti-inflammatory miR-146a was also shown to be enhanced in human asthmatic SMCs. Both miR-146a/b were found to negatively regulate pro-inflammatory cyclooxygenase-2 (COX-2) and IL-1β in human SMCs [93]. miR-146a might also contribute to tissue remodeling in the lungs because it has been shown to reduce cytokine-induced apoptosis in human bronchial epithelial cells [94]. Consistent with the findings already described in this chapter, the expression of pro-inflammatory miR-155 has been shown to be increased in asthmatic SMCs upon stimulation, and its expression positively correlates with COX-2 expression in human asthmatic SMCs [95]. Together, these studies demonstrate that the increased activation of pro-inflammatory pathways, altered expression of tight junction proteins and other changes observed in epithelial cells, SMCs and endothelium of patients with asthma may also be partially attributed to underlying aberrant miRNA expression.

microRNAs in the Regulation of Virus-Induced Asthma Exacerbation

One major cause of morbidity among asthma patients is virus-induced recurrent exacerbations, among which HRVs are the most common triggering factors [7, 96]. As asthma exacerbations are not well controlled in the severe form of the disease, studies on the molecular mechanisms of asthma exacerbation are of great importance [3]. The first implications of miRNAs in the regulation of airway responses to respiratory viruses have been published very recently. The replication of human rhinovirus HRV-1B was increased after DICER knockdown in human bronchial epithelial cells, and the transfection of specific anti-miRs for miR-128 and miR-155

increased viral replication of HRV-1B [97]. microRNAs -18a, -27a, -128, and -155 were found to be downregulated in asthmatic bronchial epithelial cells; however, manipulation of the levels of these individual microRNAs in bronchial epithelial cells did not have an effect on any pathways potentially regulating responses to HRVs, and only simultaneous knockdown of the network of microRNAs led to a significant increase in IL-8 and IL-6 expression [98].

In an early study, IL-1β-induced miR-146a was shown to reduce the levels of pro-inflammatory chemokines IL-8 and CCL5 (also known as RANTES) in human alveolar A549 epithelial cells [99], which is consistent with its strong inhibitory effect on NF-κB in other cell types [31–34]. When infecting epithelial cells, HRVs activate both NF-κB and interferon responses, whereas it has been proposed that deficient interferon induction and increased activation of NF-κB in asthmatic bronchial epithelial cells leads to aggravated inflammation and possibly to the exacerbation of asthma [100, 101]. These findings suggest an important function for microRNAs targeting NF-κB and stimulating interferon responses in protecting against exacerbation and long-lasting viral infections in patients with asthma.

miRNAs in Atopic Dermatitis, Allergic Contact Dermatitis, and Chronic Rhinosinusitis

The functions of microRNAs inatopic dermatitis (AD), allergic contact dermatitis (ACD), chronic rhinosinusitis (CRS), and allergic rhinitis (AR) have been described only in a small number of studies. miR-155 was determined to be overexpressed in skin from AD patientsmost likely due to infiltrating immune cells. The same study suggested that miR-155 influences the development of AD through the downregulation of cytotoxic T lymphocyte-associated antigen (CTLA)-4, a negative regulator of general T cell activation that also plays an important role in allergen tolerance [67, 102]. microRNA profiling of human skin challenged with DPCP and skin from a mouse model of contact dermatitis revealed that the same set of microRNAs, miR-21, miR-142-3p, miR-142-5p, and miR-223, are upregulated both in human and in mice [59]. Similar to asthmatic SMC cells, miR-146a expression was found to be increased in keratinocytes and chronic lesional skin of AD patients.

Intriguingly, the effect of miR-146a in the suppression of NF-κB pathway keratinocytes appear to largely occur due to the suppression of CARD10. In line with experiments in keratinocytes, miR-146a-deficient mice developed stronger inflammation, characterized by increased expression of genes typical in the chronic skin inflammation of AD [34]. Consistent with its binding site in IL-12p35 and inhibitory function in Th2-type airway inflammation, the third microRNA among “the three guardians,” miR-21, has been shown to suppress Th1-associated delayed-type hypersensitivity cutaneous responses in mice [54].

miR-125b has been shown to be upregulated in eosinophilic CRS with nasal polyps. miR-125b overexpression influenced IFN-α/β production, most likely through

the suppression of eukaryotic translation initiation factor 4E (EIF4E)-binding protein 4E-BP1 in airway epithelial cells [103]. miR-135a, which is reduced in a mouse model of allergic rhinitis, has been shown to correct the Th1/Th2 imbalance in the same mouse model when applied intranasally [104]. miR-143 has been shown to decrease the mRNA and protein expression levels of eotaxin and mucin 5AC (MUC5AC) in IL-13-stimulated nasal epithelial cells through direct suppression of IL-13 receptor α1 chain (IL13Rα1) [105].

An interesting aspect of micoRNAs is their relative stability in the extracellular space when compared with mRNA. Moreover, it appears that miRNAs can be actively secreted via cell-derived membrane vesicles and by protein–miRNA complexes and can also be picked up by neighboring cells [106–109]. Indeed, a recent study demonstrated that CD8+ suppressive cells are capable of producing antigen-specific antibody-coated nanovesicles containing miR-150. These nanovesicles entered into effector T cells and suppressed induced ACD and promoted antigen-specific tolerance in mice; the nanovesicles from miR-150-deficient mice did not have a suppressive effect [110]. Despite the limited number, these studies clearly demonstrate that miRNAs are involved in the regulation of allergic inflammation in the skin and sinonasal epithelial cells and strongly suggest that microRNAs represent additional means of communication between different types of cells participating in the regulation of allergic responses and the development of tolerance.

Extracellular miRNAs as Biomarkers in Allergic Diseases

Asthma is a complex disorder based on underlying cellular and molecular mechanisms and can be subcategorized into endotypes [24, 25, 85]. For better management of allergic diseases, especially severe forms of asthma, the development of endotype-specific treatment modalities is important and depends on reliable biomarkers. The microRNA expression pattern varies in different cell types and disease conditions. microRNAs are stable and can be detected in different body fluids, such as serum, urine, and saliva, and recent studies suggest that similarly to other diseases, extracellular miRNAs could be potentially used as biomarkers in airway and allergic diseases as well. In serum from patients with chronic obstructive pulmonary disease (COPD), four miRNAs (miR-20a, miR-28-3p, miR-34c-5p, and miR-100) were significantly downregulated and one (miR-7) upregulated [111].

Of ten differentially expressed miRNAs in esophageal tissue from EoE biopsies, the differential expression of miR-146a/b and miR-223 was also detected in plasma from EoE patients [57]. Bronchoalveolar lavage fluid (BALF) exosomal miRNA profiles have been found to be different in patients with mild asymptomatic asthma and healthy subjects [112]. miRNAs have also been detected in exhaled breath condensate, and some differences have been reported between asthmatic patients and control subjects [113]. miRNA expression profiles of serum and urine from 30 children with AD and 28 healthy children revealed significant upregulation of miR-203

and miR-483-5p in serum from children with AD. At the same time, miR-203 was markedly decreased in urine from children with AD compared with healthy children, and the downregulated miR-203 in urine was significantly associated with the levels of serum IgE in the AD patients [114]. Clearly, future studies are needed to determine whether extracellular miRNAs can be used as reliable biomarkers to distinguish certain endotypes of asthma and other allergic diseases.

Potential of miRNA-Based Therapeutics in Allergic Diseases

The current therapies for asthma and allergic diseases are still inefficient in controlling severe forms of these diseases, and approximately 5–10 % of all people with these disorders have inadequately controlled, severe persistent asthma or severe atopic dermatitis [3]. These asthma and allergy patients use a large proportion of public health resources, and new, more effective therapies are urgently required. One direction in the development of novel therapeutics for allergic diseases is the biological modification of immune responses for better and more individualized control [3, 115]. Indeed, there is obvious potential for miRNAs as novel target molecules for the development of biological treatment modalities. Because the functions of miRNAs are shaped by evolution, miRNA-based therapeutics would have a low likelihood of inducing toxicity, suggesting that miRNA-based therapeutics would have few side effects [116]. However, in the field of miRNA research in allergic diseases, only a limited number of people are investigating the functions of microRNAs, which is most likely due to the complexity and specificity of this research direction. Regardless, we propose that improved techniques for microRNA overexpression and inhibition, combined with increased knowledge about the biological functions of microRNAs, will lead to the development of very specific gene expression modulators that could be implemented as personalized therapeutics for allergic diseases in the future.

Based on the current knowledge of the molecular mechanisms and functions of microRNAs in allergic diseases and inflammation in general, we outline here a few ideas, whereby the targeting or overexpression of different miRNAs could possibly be utilized therapeutically in allergic tissue inflammation (Fig. 17.2). Allergic tissue inflammation in the skin, sinus, or lungs is induced by the development of a strong allergen-induced Th2-type immune response, which is strongly supported by the production of TSLP by epithelial cells [117]. As miR-375 promotes the production of TSLP [64], the inhibition of miR-375 may reduce the development of Th2-type inflammation. Suppression of the positive regulators of Th2 cell development, miR-155 [63] and miR-21 [54], in CD4+ T cells would similarly reduce the skewing of immune responses toward the Th2 direction. During the chronic phase of tissue inflammation, secondary infections and innate immune response activation in epithelial cells occurs [118], which could be suppressed with the overexpression of miR-146a/b. In the case of asthma, overexpression of the miR-17-92 cluster (87) may reduce pulmonary hypertension.

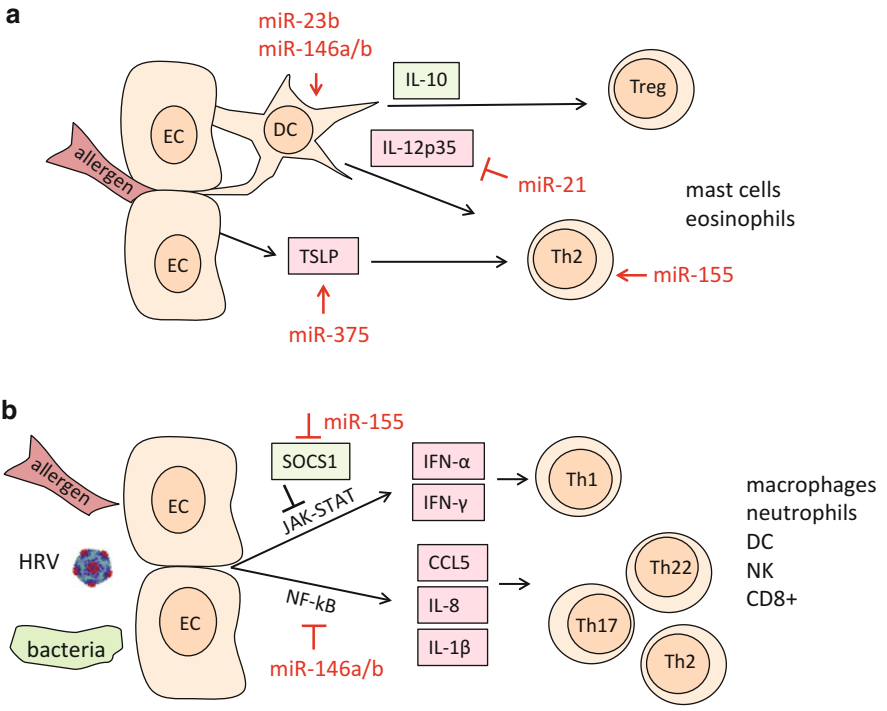

Fig. 17.2 microRNA as potential therapeutic targets in allergic inflammation. (**a**) Allergic tissue inflammation is induced by allergen-induced Th2-type immune response supported by the production of thymic stromal lymphopoietin (TSLP) by epithelial cells [117]. As miR-375 promotes the production of TSLP [64], the inhibition of miR-375 could reduce Th2 type inflammation. Suppression of the positive regulators of Th2 cell development, miR-155 [63], and miR-21, which targets IL-12p35, a positive regulator of Th1 cells [54], would also reduce Th2 type responses. Overexpression of miR-23b and miR-146a in DCs would render DCs less susceptible to the activation and might contribute to the development of Treg cells [74, 75]. (**b**) During the chronic phase of tissue inflammation, secondary infections and activation of innate immune responses in epithelial cells occurs [118], which could be suppressed by the overexpression of miR-146a/b. In HRV-induced airway inflammation or asthma exacerbation, the overexpression of miR-146a would possibly help to reduce the activation of the NF-κB pathway and accompanying inflammation, whereas the simultaneous overexpression of miR-155 would increase the interferon response and virus clearance

In HRV-induced long-term airway inflammation or asthma exacerbation, the overexpression of miR-146a would help to reduce the activation of the NF-κB pathway and accompanying inflammation, whereas the simultaneous overexpression of miR-155 would increase the interferon response and virus clearance. Overexpression of miR-23b and miR-146a in DCs would render DCs less susceptible to the activation and might contribute to the development of regulatory T cells [74, 75]. In addition, inhibition of miR-126, miR-221, miR-145, and miR-106a (Table 17.1) could possibly be used to reduce allergic inflammation in patients with asthma.

In conclusion, the roles of miRNAs in relation to allergic diseases are just beginning to be explored. Further studies are needed to reveal the full impact of miRNAs in the development of allergic diseases as well as to reveal their potential as therapeutic targets and non-invasive biomarkers in allergic conditions.

Acknowledgment This work was supported by EU structural assistance grant SARMP12219T and personal research grant PUT214 from Estonian Research Council.

References

1. Medzhitov R. Inflammation 2010: new adventures of an old flame. Cell. 2010;140:771–6.
2. Renz H, Brandtzaeg P, Hornef M. The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. Nat Rev Immunol. 2012;12:9–23.
3. Akdis CA. Therapies for allergic inflammation: refining strategies to induce tolerance. Nat Med. 2012;18:736–49.
4. Locksley RM. Asthma and allergic inflammation. Cell. 2010;140:777–83.
5. Holgate ST, Polosa R. Treatment strategies for allergy and asthma. Nat Rev Immunol. 2008;8:218–30.
6. Holt PG, Sly PD. Viral infections and atopy in asthma pathogenesis: new rationales for asthma prevention and treatment. Nat Med. 2012;18:726–35.
7. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, et al. Community study of role of viral infections in exacerbations of asthma in 9–11 year old children. BMJ. 1995;310:1225–9.
8. Jackson DJ, Johnston SL. The role of viruses in acute exacerbations of asthma. J Allergy Clin Immunol. 2010;125:1178–87; quiz 88–9.
9. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet. 2006;38:441–6.
10. Novak N, Kruse S, Kraft S, Geiger E, Kluken H, Fimmers R, et al. Dichotomic nature of atopic dermatitis reflected by combined analysis of monocyte immunophenotyping and single nucleotide polymorphisms of the interleukin-4/interleukin-13 receptor gene: the dichotomy of extrinsic and intrinsic atopic dermatitis. J Invest Dermatol. 2002;119:870–5.
11. Hershey GK, Friedrich MF, Esswein LA, Thomas ML, Chatila TA. The association of atopy with a gain-of-function mutation in the alpha subunit of the interleukin-4 receptor. N Engl J Med. 1997;337:1720–5.
12. Liu X, Nickel R, Beyer K, Wahn U, Ehrlich E, Freidhoff LR, et al. An IL13 coding region variant is associated with a high total serum IgE level and atopic dermatitis in the German multicenter atopy study (MAS-90). J Allergy Clin Immunol. 2000;106:167–70.
13. Weidinger S, Gieger C, Rodriguez E, Baurecht H, Mempel M, Klopp N, et al. Genome-wide scan on total serum IgE levels identifies FCER1A as novel susceptibility locus. PLoS Genet. 2008;4, e1000166.
14. Bieber T. Atopic dermatitis. Ann Dermatol. 2010;22:125–37.
15. Brown SJ, Sandilands A, Zhao Y, Liao H, Relton CL, Meggitt SJ, et al. Prevalent and low-frequency null mutations in the filaggrin gene are associated with early-onset and persistent atopic eczema. J Invest Dermatol. 2008;128:1591–4.
16. Vercelli D. Discovering susceptibility genes for asthma and allergy. Nat Rev Immunol. 2008;8:169–82.
17. Paternoster L, Standl M, Chen CM, Ramasamy A, Bonnelykke K, Duijts L, et al. Meta-analysis of genome-wide association studies identifies three new risk loci for atopic dermatitis. Nat Genet. 2012;44:187–92.

18. Akdis M, Trautmann A, Klunker S, Daigle I, Kucuksezer UC, Deglmann W, et al. T helper (Th) 2 predominance in atopic diseases is due to preferential apoptosis of circulating memory/effector Th1 cells. FASEB J. 2003;17:1026–35.
19. Rebane A, Zimmermann M, Aab A, Baurecht H, Koreck A, Karelson M, et al. Mechanisms of IFN-gamma-induced apoptosis of human skin keratinocytes in patients with atopic dermatitis. J Allergy Clin Immunol. 2012;129:1297–306.
20. Hamid Q, Boguniewicz M, Leung DY. Differential in situ cytokine gene expression in acute versus chronic atopic dermatitis. J Clin Invest. 1994;94:870–6.
21. Trautmann A, Akdis M, Kleemann D, Altznauer F, Simon HU, Graeve T, et al. T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. J Clin Invest. 2000;106:25–35.
22. Klunker S, Trautmann A, Akdis M, Verhagen J, Schmid-Grendelmeier P, Blaser K, et al. A second step of chemotaxis after transendothelial migration: keratinocytes undergoing apoptosis release IFN-gamma-inducible protein 10, monokine induced by IFN-gamma, and IFN-gamma-inducible alpha-chemoattractant for T cell chemotaxis toward epidermis in atopic dermatitis. J Immunol. 2003;171:1078–84.
23. Basinski TM, Holzmann D, Eiwegger T, Zimmermann M, Klunker S, Meyer N, et al. Dual nature of T cell-epithelium interaction in chronic rhinosinusitis. J Allergy Clin Immunol. 2009;124:74–80.e1–8.
24. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med. 2012;18:716–25.
25. Lotvall J, Akdis CA, Bacharier LB, Bjermer L, Casale TB, Custovic A, et al. Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. J Allergy Clin Immunol. 2011;127:355–60.
26. Rebane A, Akdis CA. MicroRNAs: essential players in the regulation of inflammation. J Allergy Clin Immunol. 2013;132:15–26.
27. Lu TX, Rothenberg ME. Diagnostic, functional, and therapeutic roles of microRNA in allergic diseases. J Allergy Clin Immunol. 2013;132:3–13; quiz 4.
28. Kumarswamy R, Volkmann I, Thum T. Regulation and function of miRNA-21 in health and disease. RNA Biol. 2011;8:706–13.
29. Iliopoulos D, Jaeger SA, Hirsch HA, Bulyk ML, Struhl K. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. Mol Cell. 2010;39:493–506.
30. Sheedy FJ, Palsson-McDermott E, Hennessy EJ, Martin C, O'Leary JJ, Ruan Q, et al. Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. Nat Immunol. 2010;11:141–7.
31. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A. 2006;103:12481–6.
32. Etzrodt M, Cortez-Retamozo V, Newton A, Zhao J, Ng A, Wildgruber M, et al. Regulation of monocyte functional heterogeneity by miR-146a and Relb. Cell Rep. 2012;1:317–24.
33. Crone SG, Jacobsen A, Federspiel B, Bardram L, Krogh A, Lund AH, et al. microRNA-146a inhibits G protein-coupled receptor-mediated activation of NF-kappaB by targeting CARD10 and COPS8 in gastric cancer. Mol Cancer. 2012;11:71.
34. Rebane A, Runnel T, Aab A, Maslovskaja J, Ruckert B, Zimmermann M, et al. MicroRNA-146a alleviates chronic skin inflammation in atopic dermatitis through suppression of innate immune responses in keratinocytes. J Allergy Clin Immunol. 2014;134:836–47.
35. Zhao JL, Rao DS, Boldin MP, Taganov KD, O'Connell RM, Baltimore D. NF-{kappa}B dysregulation in microRNA-146a-deficient mice drives the development of myeloid malignancies. Proc Natl Acad Sci U S A. 2011;108:9184–9.
36. Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. J Exp Med. 2011;208:1189–201.

37. Lu LF, Boldin MP, Chaudhry A, Lin LL, Taganov KD, Hanada T, et al. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. Cell. 2010;142:914–29.
38. Curtale G, Mirolo M, Renzi TA, Rossato M, Bazzoni F, Locati M. Negative regulation of Toll-like receptor 4 signaling by IL-10-dependent microRNA-146b. Proc Natl Acad Sci U S A. 2013;110:11499–504.
39. Xiang M, Birkbak NJ, Vafaizadeh V, Walker SR, Yeh JE, Liu S, et al. STAT3 induction of miR-146b forms a feedback loop to inhibit the NF-kappaB to IL-6 signaling axis and STAT3-driven cancer phenotypes. Sci Signal. 2014;7:11.
40. Griffiths-Jones S. miRBase: the microRNA sequence database. Methods Mol Biol. 2006;342:129–38.
41. Wang P, Hou J, Lin L, Wang C, Liu X, Li D, et al. Inducible microRNA-155 feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. J Immunol. 2010;185:6226–33.
42. O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D. Inositol phosphatase SHIP1 is a primary target of miR-155. Proc Natl Acad Sci U S A. 2009;106:7113–8.
43. Nazari-Jahantigh M, Wei Y, Noels H, Akhtar S, Zhou Z, Koenen RR, et al. MicroRNA-155 promotes atherosclerosis by repressing Bcl6 in macrophages. J Clin Invest. 2012;122:4190–202.
44. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, et al. Requirement of bic/microRNA-155 for normal immune function. Science. 2007;316:608–11.
45. O'Connell RM, Kahn D, Gibson WS, Round JL, Scholz RL, Chaudhuri AA, et al. MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. Immunity. 2010;33:607–19.
46. Boldin MP, Baltimore D. MicroRNAs, new effectors and regulators of NF-kappaB. Immunol Rev. 2012;246:205–20.
47. Baumjohann D, Ansel KM. MicroRNA-mediated regulation of T helper cell differentiation and plasticity. Nat Rev Immunol. 2013;13:666–78.
48. de Yebenes VG, Bartolome-Izquierdo N, Ramiro AR. Regulation of B-cell development and function by microRNAs. Immunol Rev. 2013;253:25–39.
49. Schmidt-Weber CB, Akdis M, Akdis CA. TH17 cells in the big picture of immunology. J Allergy Clin Immunol. 2007;120:247–54.
50. Scott JT, Turner C, Mutapi F, Woolhouse ME, Chandiwana SK, Mduluza T, et al. Dissociation of interleukin-4 and interleukin-5 production following treatment for Schistosoma haematobium infection in humans. Parasite Immunol. 2000;22:341–8.
51. Mary C, Auriault V, Faugere B, Dessein AJ. Control of Leishmania infantum infection is associated with CD8(+) and gamma interferon- and interleukin-5-producing CD4(+) antigen-specific T cells. Infect Immun. 1999;67:5559–66.
52. Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, et al. Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. J Allergy Clin Immunol. 2011;127:701–21.e1–70.
53. Lu TX, Munitz A, Rothenberg ME. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. J Immunol. 2009;182:4994–5002.
54. Lu TX, Hartner J, Lim EJ, Fabry V, Mingler MK, Cole ET, et al. MicroRNA-21 limits in vivo immune response-mediated activation of the IL-12/IFN-gamma pathway, Th1 polarization, and the severity of delayed-type hypersensitivity. J Immunol. 2011;187:3362–73.
55. Case SR, Martin RJ, Jiang D, Minor MN, Chu HW. MicroRNA-21 inhibits toll-like receptor 2 agonist-induced lung inflammation in mice. Exp Lung Res. 2011;37:500–8.
56. Chen RF, Huang HC, Ou CY, Hsu TY, Chuang H, Chang JC, et al. MicroRNA-21 expression in neonatal blood associated with antenatal immunoglobulin E production and development of allergic rhinitis. Clini Exp Allergy. 2010;40:1482–90.
57. Lu TX, Sherrill JD, Wen T, Plassard AJ, Besse JA, Abonia JP, et al. MicroRNA signature in patients with eosinophilic esophagitis, reversibility with glucocorticoids, and assessment as disease biomarkers. J Allergy Clin Immunol. 2012;129:1064–75.e9.

58. Lu TX, Lim EJ, Wen T, Plassard AJ, Hogan SP, Martin LJ, et al. MiR-375 is downregulated in epithelial cells after IL-13 stimulation and regulates an IL-13-induced epithelial transcriptome. Mucosal Immunol. 2012;5:388–96.
59. Vennegaard MT, Bonefeld CM, Hagedorn PH, Bangsgaard N, Lovendorf MB, Odum N, et al. Allergic contact dermatitis induces upregulation of identical microRNAs in humans and mice. Contact Dermatitis. 2012;67:298–305.
60. Kumar M, Ahmad T, Sharma A, Mabalirajan U, Kulshreshtha A, Agrawal A, et al. Let-7 microRNA-mediated regulation of IL-13 and allergic airway inflammation. J Allergy Clin Immunol. 2011.
61. Polikepahad S, Knight JM, Naghavi AO, Oplt T, Creighton CJ, Shaw C, et al. Proinflammatory role for let-7 microRNAS in experimental asthma. J Biol Chem. 2010;285:30139–49.
62. Martinez-Nunez RT, Louafi F, Sanchez-Elsner T. The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Ralpha1). J Biol Chem. 2011;286:1786–94.
63. Okoye IS, Czieso S, Ktistaki E, Roderick K, Coomes SM, Pelly VS, et al. Transcriptomics identified a critical role for Th2 cell-intrinsic miR-155 in mediating allergy and antihelminth immunity. Proc Natl Acad Sci U S A. 2014;111:E3081–90.
64. Biton M, Levin A, Slyper M, Alkalay I, Horwitz E, Mor H, et al. Epithelial microRNAs regulate gut mucosal immunity via epithelium-T cell crosstalk. Nat Immunol. 2011;12:239–46.
65. Bleck B, Grunig G, Chiu A, Liu M, Gordon T, Kazeros A, et al. MicroRNA-375 regulation of thymic stromal lymphopoietin by diesel exhaust particles and ambient particulate matter in human bronchial epithelial cells. J Immunol. 2013;190:3757–63.
66. Akdis M, Palomares O, van de Veen W, van Splunter M, Akdis CA. TH17 and TH22 cells: a confusion of antimicrobial response with tissue inflammation versus protection. J Allergy Clin Immunol. 2012;129:1438–49; quiz50–1.
67. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med. 2004;199:1567–75.
68. Takahashi H, Kanno T, Nakayamada S, Hirahara K, Sciume G, Muljo SA, et al. TGF-beta and retinoic acid induce the microRNA miR-10a, which targets Bcl-6 and constrains the plasticity of helper T cells. Nat Immunol. 2012;13:587–95.
69. Warth SC, Hoefig KP, Hiekel A, Schallenberg S, Jovanovic K, Klein L, et al. Induced miR-99a expression represses Mtor cooperatively with miR-150 to promote regulatory T-cell differentiation. EMBO J. 2015.
70. Lambrecht BN, Hammad H. The immunology of asthma. Nat Immunol. 2015;16:45–56.
71. Amu S, Saunders SP, Kronenberg M, Mangan NE, Atzberger A, Fallon PG. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. J Allergy Clin Immunol. 2010;125:1114–24.e8.
72. Bousquet J, Anto JM, Demoly P, Schunemann HJ, Togias A, Akdis M, et al. Severe chronic allergic (and related) diseases: a uniform approach—a MeDALL—GA2LEN—ARIA position paper. Int Arch Allergy Immunol. 2012;158:216–31.
73. Hrusch CL, Tjota MY, Sperling AI. The role of dendritic cells and monocytes in the maintenance and loss of respiratory tolerance. Curr Allergy Asthma Rep. 2015;15:494.
74. Zheng J, Jiang HY, Li J, Tang HC, Zhang XM, Wang XR, et al. MicroRNA-23b promotes tolerogenic properties of dendritic cells in vitro through inhibiting Notch1/NF-kappaB signalling pathways. Allergy. 2012;67:362–70.
75. Jurkin J, Schichl YM, Koeffel R, Bauer T, Richter S, Konradi S, et al. miR-146a is differentially expressed by myeloid dendritic cell subsets and desensitizes cells to TLR2-dependent activation. J Immunol. 2010;184:4955–65.
76. Feng MJ, Shi F, Qiu C, Peng WK. MicroRNA-181a, -146a and -146b in spleen CD4+ T lymphocytes play proinflammatory roles in a murine model of asthma. Int Immunopharmacol. 2012;13:347–53.

77. Malmhall C, Alawieh S, Lu Y, Sjostrand M, Bossios A, Eldh M, et al. MicroRNA-155 is essential for T2-mediated allergen-induced eosinophilic inflammation in the lung. J Allergy Clin Immunol. 2013.
78. Mattes J, Collison A, Plank M, Phipps S, Foster PS. Antagonism of microRNA-126 suppresses the effector function of TH2 cells and the development of allergic airways disease. Proc Natl Acad Sci U S A. 2009;106:18704–9.
79. Collison A, Herbert C, Siegle JS, Mattes J, Foster PS, Kumar RK. Altered expression of microRNA in the airway wall in chronic asthma: miR-126 as a potential therapeutic target. BMC Pulm Med. 2011;11:29.
80. Collison A, Mattes J, Plank M, Foster PS. Inhibition of house dust mite-induced allergic airways disease by antagonism of microRNA-145 is comparable to glucocorticoid treatment. J Allergy Clin Immunol. 2011;128:160–7.e4.
81. Sharma A, Kumar M, Ahmad T, Mabalirajan U, Aich J, Agrawal A, et al. Antagonism of mmu-mir-106a attenuates asthma features in allergic murine model. J Appl Physiol. 2012;113:459–64.
82. Qin HB, Xu B, Mei JJ, Li D, Liu JJ, Zhao DY, et al. Inhibition of miRNA-221 suppresses the airway inflammation in asthma. Inflammation. 2012;35:1595–9.
83. Montagner S, Orlandi EM, Merante S, Monticelli S. The role of miRNAs in mast cells and other innate immune cells. Immunol Rev. 2013;253:12–24.
84. Mayoral RJ, Pipkin ME, Pachkov M, van Nimwegen E, Rao A, Monticelli S. MicroRNA-221-222 regulate the cell cycle in mast cells. J Immunol. 2009;182:433–45.
85. Agache I, Akdis C, Jutel M, Virchow JC. Untangling asthma phenotypes and endotypes. Allergy. 2012;67:835–46.
86. Williams AE, Larner-Svensson H, Perry MM, Campbell GA, Herrick SE, Adcock IM, et al. MicroRNA expression profiling in mild asthmatic human airways and effect of corticosteroid therapy. PLoS One. 2009;4, e5889.
87. Jardim MJ, Dailey L, Silbajoris R, Diaz-Sanchez D. Distinct microRNA expression in human airway cells of asthmatic donors identifies a novel asthma-associated gene. Am J Respir Cell Mol Biol. 2012;47:536–42.
88. Takyar S, Vasavada H, Zhang JG, Ahangari F, Niu N, Liu Q, et al. VEGF controls lung Th2 inflammation via the miR-1-Mpl (myeloproliferative leukemia virus oncogene)-P-selectin axis. J Exp Med. 2013;210:1993–2010.
89. Chiba Y, Misawa M. MicroRNAs and their therapeutic potential for human diseases: MiR-133a and bronchial smooth muscle hyperresponsiveness in asthma. J Pharmacol Sci. 2010;114:264–8.
90. Jude JA, Dileepan M, Subramanian S, Solway J, Panettieri Jr RA, Walseth TF, et al. miR-140-3p regulation of TNF-alpha-induced CD38 expression in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol. 2012;303:L460–8.
91. Chen T, Zhou G, Zhou Q, Tang H, Ibe JC, Cheng H, et al. Loss of miR-17~92 in smooth muscle cells attenuates experimental pulmonary hypertension via induction of PDLIM5. Am J Respir Crit Care Med. 2015;191(6):678–92.
92. Dileepan M, Jude JA, Rao SP, Walseth TF, Panettieri RA, Subramanian S, et al. MicroRNA-708 regulates CD38 expression through signaling pathways JNK MAP kinase and PTEN/AKT in human airway smooth muscle cells. Respir Res. 2014;15:107.
93. Comer BS, Camoretti-Mercado B, Kogut PC, Halayko AJ, Solway J, Gerthoffer WT. MicroRNA-146a and microRNA-146b expression and anti-inflammatory function in human airway smooth muscle. Am J Physiol Lung Cell Mol Physiol. 2014;307:L727–34.
94. Liu X, Nelson A, Wang X, Kanaji N, Kim M, Sato T, et al. MicroRNA-146a modulates human bronchial epithelial cell survival in response to the cytokine-induced apoptosis. Biochem Biophys Res Commun. 2009;380:177–82.
95. Comer BS, Camoretti-Mercado B, Kogut PC, Halayko AJ, Solway J, Gerthoffer WT. Cyclooxygenase-2 and microRNA-155 expression are elevated in asthmatic airway smooth muscle cells. Am J Respir Cell Mol Biol. 2014;52(4):438–47.

96. Nicholson KG, Kent J, Ireland DC. Respiratory viruses and exacerbations of asthma in adults. BMJ. 1993;307:982–6.
97. Bondanese VP, Francisco-Garcia A, Bedke N, Davies DE, Sanchez-Elsner T. Identification of host miRNAs that may limit human rhinovirus replication. World J Biol Chem. 2014;5:437–56.
98. Martinez-Nunez RT, Bondanese VP, Louafi F, Francisco-Garcia AS, Rupani H, Bedke N, et al. A microRNA network dysregulated in asthma controls IL-6 production in bronchial epithelial cells. PLoS One. 2014;9, e111659.
99. Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larner-Svensson HM, Lindsay MA. Rapid changes in microRNA-146a expression negatively regulate the IL-1beta-induced inflammatory response in human lung alveolar epithelial cells. J Immunol. 2008;180:5689–98.
100. Edwards MR, Regamey N, Vareille M, Kieninger E, Gupta A, Shoemark A, et al. Impaired innate interferon induction in severe therapy resistant atopic asthmatic children. Mucosal Immunol. 2013;6:797–806.
101. Bartlett NW, Slater L, Glanville N, Haas JJ, Caramori G, Casolari P, et al. Defining critical roles for NF-kappaB p65 and type I interferon in innate immunity to rhinovirus. EMBO Mol Med. 2012;4:1244–60.
102. Sonkoly E, Janson P, Majuri ML, Savinko T, Fyhrquist N, Eidsmo L, et al. MiR-155 is overexpressed in patients with atopic dermatitis and modulates T-cell proliferative responses by targeting cytotoxic T lymphocyte-associated antigen 4. J Allergy Clin Immunol. 2010;126:581–9.e1–20.
103. Zhang XH, Zhang YN, Li HB, Hu CY, Wang N, Cao PP, et al. Overexpression of miR-125b, a novel regulator of innate immunity, in eosinophilic chronic rhinosinusitis with nasal polyps. Am J Respir Crit Care Med. 2012;185:140–51.
104. Luo Y, Deng Y, Tao Z, Chen S, Xiao B, Ren J, et al. Regulatory effect of microRNA-135a on the Th1/Th2 imbalance in a murine model of allergic rhinitis. Exp Ther Med. 2014;8:1105–10.
105. Teng Y, Zhang R, Liu C, Zhou L, Wang H, Zhuang W, et al. miR-143 inhibits interleukin-13-induced inflammatory cytokine and mucus production in nasal epithelial cells from allergic rhinitis patients by targeting IL13Ralpha1. Biochem Biophys Res Commun. 2015;457:58–64.
106. Redis RS, Calin S, Yang Y, You MJ, Calin GA. Cell-to-cell miRNA transfer: from body homeostasis to therapy. Pharmacol Ther. 2012;136:169–74.
107. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol. 2011;13:423–33.
108. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A. 2011;108:5003–8.
109. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. Nucleic Acids Res. 2011;39:7223–33.
110. Bryniarski K, Ptak W, Jayakumar A, Pullmann K, Caplan MJ, Chairoungdua A, et al. Antigen-specific, antibody-coated, exosome-like nanovesicles deliver suppressor T-cell microRNA-150 to effector T cells to inhibit contact sensitivity. J Allergy Clin Immunol. 2013;132:170–81.
111. Akbas F, Coskunpinar E, Aynaci E, Oltulu YM, Yildiz P. Analysis of serum micro-RNAs as potential biomarker in chronic obstructive pulmonary disease. Exp Lung Res. 2012;38:286–94.
112. Levanen B, Bhakta NR, Torregrosa Paredes P, Barbeau R, Hiltbrunner S, Pollack JL, et al. Altered microRNA profiles in bronchoalveolar lavage fluid exosomes in asthmatic patients. J Allergy Clin Immunol. 2013;131:894–903.

113. Sinha A, Yadav AK, Chakraborty S, Kabra SK, Lodha R, Kumar M, et al. Exosome-enclosed microRNAs in exhaled breath hold potential for biomarker discovery in patients with pulmonary diseases. J Allergy Clin Immunol. 2013;132:219–22.
114. Lv Y, Qi R, Xu J, Di Z, Zheng H, Huo W, et al. Profiling of serum and urinary microRNAs in children with atopic dermatitis. PLoS One. 2014;9, e115448.
115. Akdis M, Akdis CA. Therapeutic manipulation of immune tolerance in allergic disease. Nat Rev Drug Discov. 2009;8:645–60.
116. Kanasty RL, Whitehead KA, Vegas AJ, Anderson DG. Action and reaction: the biological response to siRNA and its delivery vehicles. Mol Ther. 2012;20:513–24.
117. Robinson DS. The role of the T cell in asthma. J Allergy Clin Immunol. 2010;126:1081–91; quiz 92–3.
118. Holtzman MJ. Asthma as a chronic disease of the innate and adaptive immune systems responding to viruses and allergens. J Clin Invest. 2012;122:2741–8.
119. Tsitsiou E, Williams AE, Moschos SA, Patel K, Rossios C, Jiang X, et al. Transcriptome analysis shows activation of circulating CD8+ T cells in patients with severe asthma. J Allergy Clin Immunol. 2012;129:95–103.

Chapter 18
microRNA in Human Reproduction

Iris Eisenberg, Noora Kotaja, Debra Goldman-Wohl, and Tal Imbar

Abstract microRNAs constitute a large family of approximately 21-nucleotide-long, noncoding RNAs. They emerged more than 20 years ago as key posttranscriptional regulators of gene expression. The regulatory role of these small RNA molecules has recently begun to be explored in the human reproductive system. microRNAs have been shown to play an important role in control of reproductive functions, especially in the processes of oocyte maturation, folliculogenesis, corpus luteum function, implantation, and early embryonic development. Knockout of Dicer, the cytoplasmic enzyme that cleaves the pre-miRNA to its mature form, results in postimplantation embryonic lethality in several animal models, attributing to these small RNA vital functions in reproduction and development. Another intriguing characteristic of microRNAs is their presence in body fluids in a remarkably stable form that is protected from endogenous RNase activity.

In this chapter we will describe the current knowledge on microRNAs, specifically relating to human gonadal cells. We will focus on their role in the ovarian physiologic process and ovulation dysfunction, regulation of spermatogenesis and male fertility, and putative involvement in human normal and aberrant trophoblast differentiation and invasion through the process of placentation.

Keywords Ovulation • Corpus luteum • Spermatogenesis • Biomarkers • Placenta

I. Eisenberg • D. Goldman-Wohl • T. Imbar (✉)
Department of Obstetrics and Gynecology, The Magda and Richard Hoffman Center for Human Placenta Research, Hadassah Mt. Scopus—Hebrew University Medical Center, Jerusalem, Israel
e-mail: talim@hadassah.org.il

N. Kotaja
Department of Physiology, Institute of Biomedicine, University of Turku, Turku, Finland

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_18

Introduction

Ovulation of a mature and viable oocyte and the formation of a functional corpus luteum (CL) are essential for establishment of pregnancy. These events are preceded by a highly orchestrated series of growth and developmental events in the periovulatory follicle that ultimately signals the preovulatory surge of luteinizing hormone (LH) resulting in the initiation of ovulation and CL formation. This is a dynamic and highly regulated process that requires the coordinated actions of multiple tissues and organ systems (e.g., hypothalamus, pituitary, ovary, and reproductive tract) to develop a fertilizable gamete, as well as provide a suitable environment for fertilization and subsequent fetal development. To attain this optimal environment, the female reproductive system must be highly responsive to subtle changes in hormones and other external cues.

Posttranscriptional gene regulation by microRNAs (miRNAs) plays a key role during tissue development and differentiation. miRNAs target gene expression stability through transcriptional repression or degradation and may play an important role in control of reproductive endocrine functions especially the process of oocyte maturation and folliculogenesis. Mammalian gonads which exhibit strictly regulated spatiotemporal gene expression patterns are also known to express unique sets of miRNAs and genes involved in the miRNA biogenetic pathway. miRNAs are also associated with functional regulation of gonadal somatic cells, granulosa cells/cumulus cells, in steroid synthesis. Several functional studies have shown the role of miRNAs in pathology, fertility, and development [1–4]. Functional studies involving inhibition of miRNA biogenesis have shown the occurrence of developmental arrest and female infertility in various species [5]. Continuous advances in our understanding of the role and function of miRNAs in ovarian cells will lead to a better understanding of the control of follicular and luteal development in normal and pathological conditions.

Male gametes are produced inside mammalian testes during spermatogenesis that includes three phases: (1) mitotic proliferation of spermatogonia, (2) meiosis of spermatocytes, and (3) haploid differentiation of spermatids. The progress of spermatogenesis is governed by phase-specific gene expression patterns for both protein coding mRNAs and noncoding RNAs (ncRNAs). Also miRNAs are expressed in a cell type-specific manner during spermatogenesis and participate in the control of the specific steps of male germ cell differentiation. Genetically modified mouse models to delete the miRNA processing enzymes have revealed the importance of miRNA pathway for normal spermatogenesis and male fertility. Functional studies have elucidated the roles of specific miRNAs in distinct cell types. Furthermore, expression profiles of miRNAs have been explored in human testis, spermatozoa, and seminal plasma, and it has been shown that spermatozoal or seminal plasma miRNAs may serve as potential biomarkers for male infertility.

Although embryo implantation is essential for human survival, it remains an enigmatic biological phenomenon. Following fertilization, the resulting blastocyst must signal its presence to the mother, attach to the luminal epithelium of the endometrium, and embed into the decidualising stroma. Subsequent placental

development requires remodeling of maternal blood vessels by trophoblast cells that invade deep into the decidua. Failure in these very early stages can compromise fetal development, resulting in diseases of pregnancy such as intrauterine growth restriction or preeclampsia which can also impact on health in adulthood. A different chapter in this book is dealing with the role of miRNAs during early implantation. In this chapter we focus in the mature product of implantation, the placenta. We describe several topics of investigation dealing with miRNA-mediated regulation of the placenta during pregnancy. This miRNA function remains poorly investigated although several independent processes associated with placenta development have been shown to be miRNA-regulated. miRNA may reflect disorders not yet detectable with other methods and contribute to understanding the underlying pathological mechanisms.

microRNA in the Ovary

Dynamically regulated sequential recruitment, selection and growth of the follicles, atresia, ovulation, and luteolysis in the ovary are under control of closely coordinated endocrine and paracrine factors which are controlled by tightly regulated expression and interaction of a multitude of genes in the ovary. miRNAs constitute the most abundant class of small RNAs in the ovary [6].

As one of the major classes of gene regulators these miRNA molecules are suggested to be involved in the regulation of genes in the ovary. The miRNA population in the ovaries was identified in human and other species using microarrays, high throughput qPCR, and next generation sequencing techniques [7–14]. These studies revealed preferential expression of several miRNAs in the ovary of different species, suggesting that they may have an important role in ovarian functions. Regardless of species, let-7 family, miR-21, miR-99a, miR-125b, miR-126, miR-143, miR-145, and miR-199b were found to be the most commonly abundant miRNAs populations in the ovary [15]. Bioinformatics prediction, screening, and Gene Ontology analysis of these predominantly expressed miRNAs target genes in the mammalian ovary has identified several biological processes and pathways or molecular networks including cell cycle regulation, cell death, cell to cell signaling, cellular growth, development and proliferation, endocrine system disorder, and different pathways underlying the ovarian functions [13].

Role of Dicer in the Oocyte

Studies of the role of miRNAs in ovarian function have been highlighted primarily through Dicer [16]. Dicer is an RNase III cytoplasmatic enzyme required for processing small regulatory RNA, including miRNAs, which originate from endogenous single-stranded hairpin precursors to its mature form [17–20]. As the function of

Dicer and its products (miRNAs and siRNAs) are studied in the female reproductive tract, vital roles for such posttranscriptional gene regulation in female fertility are revealed [21]. Oocytes and fertilized eggs contain 10–15-fold higher levels of Dicer transcripts than any other cells and/or tissues (BioGPS; http://biogps.gnf.org/) [22] and are one of few known mammalian cells and/or tissues in which Dicer expression is regulated [23].

Expression of the Dicer transcript remains steady in the growing mouse oocyte during folliculogenesis [24] and through the germinal vesicle and metaphase II stages [25, 26]. After fertilization, the amount of Dicer mRNA decreases by approximately half and remains low in the two-cell embryo through the blastocyst stage [25, 26]. Expression of total miRNA during this same period is highest in the mature oocyte and one-cell zygote before decreasing by half in the two-cell embryo [27]. Similar to the degradation of maternal messenger RNA [28], there is a significant global loss of maternal miRNAs between the one- and two-cell stages of development; de novo synthesis of miRNAs commences at the two-cell stage. In the mice, knockout (KO) of Dicer results in postimplantation embryonic lethality [5].

Using a site-specific recombinase technology, mice with a conditional knockdown (cKO) of Dicer revealed a reduction in ovarian weight and lower ovulation rates [29]. Dicer1 cKO mouse was shown to be infertile due to multiple defects in ovarian functions including abnormal estrous cycles, shorter estrus and longer metestrous, paratubal cyst, and abnormal response to gonadotropin with ultimate problem in ovulation [30]. Likewise, Dicer1 conditional knockout mouse in ovarian granulose cells led to an increased primordial follicle pool endowment accelerated early follicle recruitment, and more follicle degeneration in the cKO ovaries. The same study has reported significant differences in the expression of some follicle development-related genes between cKO and WT mouse ovaries, such as Amh, Inhba, Cyp17a1, Cyp19a1, Zps, Gdf9, and Bmp15, suggesting the important function of miRNAs in regulating ovarian gene expression [31].

Studies in rodent oocytes using ovarian specific Dicer knockouts have shown no effect on early folliculogenesis and oocyte development. Examining the KO oocytes revealed an impaired ability to extrude a polar body after mating. Immunostaining indicated multiple spindles and chromatin condensation defects [26, 27, 32]. These data suggest that meiotic defects arise from the ooplasm of the oocyte and not the germinal vesicle. These, loss of Dicer expression, experiments indicate that Dicer affects ovulation rates, probably by influencing the total number of periovulatory follicles that achieve proper development and/or by affecting the ability of follicles to ovulate (see Chap. 8 of the volume “microRNA and Cancer” for a detailed discussion on miRNA and ovarian cancer).

Role of Dicer in Cumulus Cells

Dicer expression in other somatic tissues of the ovary (theca cells, corpus luteum and interstitium) has not been examined directly. The levels of Dicer in granulosa cells did not change when examined before and after the LH surge [33]. Functional

deletion studies of Dicer, points to an important role for miRNA in ovarian function and female fertility [29, 30, 34–36]. Otsuka and colleagues [34] created a general hypomorphic mutation (Dicerhypo; ~75 % reduction in Dicer protein) using a gene-trap method and observed that Dicerhypo females were infertile because of luteal deficiency. Transplantation of wild-type ovaries into Dicerhypo females restored fertility, indicating that loss of fertility was due to an ovarian defect. Serum progesterone levels in Dicerhypo mice remained low after mating, and histological analyses of ovaries revealed a lack of luteal tissue vascularization. This indicates that the physiological process in the development and function of the ovarian corpus luteum requires Dicer1 function and specifically the mechanism of miRNAs-mediated regulation. The loss of Dicer expression in ovarian granulosa cells reduced natural, equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) stimulated ovulation rates when compared with wild type mice [29, 30]. Increased numbers of atretic follicles and trapped oocytes in luteinizing follicles were also observed.

miRNAs in the Ovarian Corpus Luteum

Several studies have identified miRNA population specifically associated with the development of follicles and corpora lutea [33, 37, 38]. A study of miRNAs expression in mouse mural granulosa cells collected before and after an ovulatory dose of hCG identified that miR-132 and miR-212, two tandemly expressed miRNAs that target the same transcripts, are highly upregulated following LH/hCG induction [33]. It is suggested that miR-132 and miR-212 play an important role as posttranscriptional regulators in granulosa cells, as computational analysis has identified 77 putative mRNA as potential targets of miR-212, and miR-132 in granulosa cells [33]. C terminal binding protein 1 (CTBP1) is a known target of miR-132, and the gene product acts as a co-repressor of nuclear receptor genes. Interestingly, knockdown of both miR-212/miR-132 resulted in decreased protein levels of CTBP1 but with no change in mRNA levels [33].

A recent study described the mechanism underlying the up-regulation of FSH-subunit synthesis by a GnRH dependant increase in miR-132 and miR-212 in gonadotropin producing cells [39]. It was shown that the miR-132/212-mediated action of GnRH peptide involved a posttranscriptional decrease of Sirtuin 1 (SIRT1) deacetylase. The lower level of SIRT1 deacetylase correlated with an increase in the acetylated form of Forkhead BoxO1 (FOXO1), a transcriptional repressor of *Fshb* gene. A different study investigating the role of miRNAs in human granulosa cells (hGC) used 80 individual synthetic miRNA precursors that mimic endogenous miRNAs and transfected them into cultured primary ovarian granulosa cells [37]. This transfection of cultured hGC with selected miRNA constructs (miR-108, miR-7, miR-9, miR-105, miR-128, miR-132, miR-141, miR-142, miR-152, miR-188, and miR-191) resulted in a significant increase in the percentage of cells containing PCNA, a cell proliferation marker. Similarly, miR-15a, miR-96, miR-92, miR-124,

miR-18, miR-29a, miR-125a, miR-136, miR-147, miR-183, and miR-32 were found to promote up to twofold accumulation of Bax, a pro-apoptotic marker, in hGC. A different study used a genome scale screen approach in order to identify miRNAs controlling human ovarian cell steroidogenesis [37]. The authors transfected 80 different gene constructs encoding human pre-miRNAs into primary granulosa cells from women undergoing ovariectomy because of non-metastatic cancer of the uterine cervix. 36 out of 80 tested miRNA constructs resulted in inhibition of progesterone release and 10 miRNAs promoted progesterone release. 57 miRNAs were found to inhibit testosterone release and 51 suppressed estradiol release.

A study in rodents revealed a bi-phase regulation of miRNAs by FSH [40]. After 12 h of FSH exposure, 17 miRNAs were up regulated and 14 miRNAs were down regulated. A significant decrease in the expression levels of miR-29a and miR-30d during the first 12 h post FSH exposure was observed. However, FSH induced a two- and threefold increase in the expression of miR-29a and miR-30d at 48 h post-FSH exposure, respectively. These two miRNAs could be involved in the fine-tuning of FSH mediated granulose cell function. In this same study FSH up regulated progesterone levels in the medium at 12 h [40], suggesting that miRNAs may play a role mediating changes in gene expression and thus hormone production in granulosa cells following FSH exposure. In addition, it was demonstrated that granulosa cells collected, immediately before and 4 h after the ovulatory surge of LH/hCG, exhibit differential miRNA expression patterns [41], suggesting a role for miRNAs in ovulation. In vitro over expression and inhibition experiments revealed that aromatase gene expression (CYP19A1) and therefore estradiol production, by porcine granulosa cells is post transcriptionally downregulated by miR-378 [42]. These results suggest that miRNA may play a key role in the fine tuning of gene expression cascade, to allow the processes of ovulation, differentiation of luteal cells and corpus luteum function to occur in the mammalian ovary. Most recently, *in vitro* studies have shown evidence for posttranscriptional regulation of Progesterone receptor (PGR) by miR-378-3p [43]. miRNA-378-3p decreased protein levels and mRNA levels of PGR via direct targeting its 3′UTR but also led to lower expression levels of ADAMTS1, CTSL1 and PPARG transcripts which are known to be regulated by PGR and are important for follicular maturation and remodeling.

miRNAs in Hypothalamic-Pituitary-Ovarian Axis

Ovarian follicle development is regulated by the hypothalamic-pituitary-ovarian axis, in which gonadotropin-releasing hormone (GnRH) controls the release of the gonadotropic hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Ovarian steroids exert both negative and positive regulatory effects on GnRH hypothalamic secretion. Specific deletion of *Dicer* in gonadotropin producing cells results in altered gonadotropin homeostasis leading to reproductive dysfunction [44]. Those mutants lacking *Dicer* in their gonadotropin producing cells were rescued with supplementation of exogenous hormones. This work confirmed

the assumption that the fertility defects which evolved from reduced *Dicer* activity are secondary to suppressed gonadotropins production and action [44]. Thus, the identification of specific miRNAs which selectively target individual gonadotropin subunits would provide novel *in vivo* therapeutic tool and provide a feasible approach to manipulate pituitary gonadotropin secretion. A different study demonstrated that miRNA-200b and miRNA-429, members of the miRNA 200 family, strongly affect ovulation in female mice. Knockout mice lacking miRNA-200b and -429 displayed a defective hypothalamic-pituitary-ovarian axis and failed to ovulate [45].

The involvement of the miRNA 200 family in humans has been related to tumorogenesis, particularly in the epithelial to mesenchymal transition [46]. This process is an important component of corpus luteum formation, especially during the intense angiogenesis occurring after the LH signal of ovulation is attained. Recently our lab measured miRNA-200b and -429 expression in hGCs and in the serum in the different phases of the ovulation cycle. We found that these miRNAs are expressed in the serum of women. Their expression changes during the phases of the normal ovarian cycle. We also discovered a distinct difference in the expression pattern of the two miRNAs in the serum of anovulatory women during early follicular phase. miRNA-200b and -429 in anovulatory women were highly expressed and their levels dropped after gonadotropin stimulation immediately post the induced LH surge to levels comparable to those found in ovulatory women (In preparation for publication). Our results correlate with the study in mice [45] which examined the expression of these miRNAs in the mouse pituitary. In this experiment the formation of a miRNA-disrupted gene knockout to miRNA-200b and -429, caused the female mice to be sterile and regain their fertility only after superovulation with exogenous gonadotropins. These findings elaborate the hypothesis that these miRNAs might act as regulators of hormonal pituitary function and with dysfunctional central effect, such as in PCOS, the ovarian follicles will not ovulate naturally.

miRNAs and Ovarian Dysfunction

Given their important roles in normal physiology, it is no surprise that miRNAs have come under close scrutiny in a variety of diseases. miRNAs have been shown to play contributory roles in cancer [47–51], heart disease [52, 53], infectious disease [54–57], and other medical conditions [58–60]. Polycystic ovary syndrome (PCOS) is one of the most common endocrine-metabolic disorders in women of reproductive age [61]. It has been proposed that PCOS results from abnormal regulation of steroidogenesis and specifically from androgen secretion by the ovary [62].

Expression of miRNAs in granulosa cells may have a direct regulatory effect on the expression of specific genes involved in folliculogenesis and ovarian steroidogenesis. The involvement of miRNAs in the control of the release of the main ovarian steroids progesterone and estradiol is the key question. The differences in microRNA expression may explain the aberrant follicular development and aid in identifying pathways associated with this phenotype. Many biological mechanisms

are regulated by microRNA, but only very few studies have investigated the role of miRNAs in PCOS (Tables 18.1 and 18.2). Two studies were the first to identify miRNAs in micro-vesicles and in supernatant of human follicular fluid from patients with PCOS [63, 64]. Both investigators found differentially expressed miRNAs in women with PCOS compared to a control group. The first group investigated a sub set of 7 miRNAs (miR-132, 320, 24, 520c-3p, 193b, 483-5p, and 222) in the follicular fluid of women with PCOS. The 7 miRNAs were chosen secondary to their association with steroidogenesis and two were found to have significantly decreased expression in the PCOS group (miR-132 and 320). miR-132, miR-320, miR-520c-3p, and miR-222 regulate the concentration of estradiol and miR-24, miR-193b, and miR-483-5p regulate progesterone concentrations (Table 18.2).

A recent study demonstrated that the Notch3 and MAPK3 are targeted by miR-483-5p which is down regulated in granulosa cells from PCOS patients [65] (Table 18.1). It had been shown that miRNA-320 is mainly expressed in GCs and oocytes of mouse ovarian follicles in the time of follicular development. Overexpression of miR-320 inhibited estradiol synthesis and proliferation of GCs through targeting E2F1 and SF-1. E2F1/SF-1 mediated miR-320-induced suppression of GC proliferation and of GC steroidogenesis. FSH down-regulated the expression of miR-320 and regulated the function of miR-320 in mouse GCs. miR-383 promoted the expression of miR-320 and enhanced miR-320-mediated suppression of GC proliferation [66].

The mechanism by which those miRNAs regulate estradiol and progesterone expression and corpus luteum secretion is still unknown. Bioinformatic analysis showed that the most highly expressed miRNAs targeted genes are associated with reproductive, endocrine, and metabolic processes. The second group also identified 5 miRNAs (hsa-miR-9, 18b, 32, 34c, and 135a) with significant overexpression in the PCOS group versus the control group [63] (Table 18.2). Three potential miRNA-target genes had significantly decreased expression in the women with PCOS: insulin receptor substrate 2 (IRS2), Synaptotagmin 1(SYT1) and interleukin 8 (IL8). Mice lacking IRS2 demonstrate dysregulation of the estrous cycle, anovulation, infertility, and insulin resistance, similar to women with PCOS [67]; IL8 is implicated in steroid synthesis in the late follicular and ovulatory follicle in both bovine and humans [68]. All three have functions related to the PCOS phenotype including roles in carbohydrate metabolism and beta cell function, cell–cell communication, and steroid synthesis. The level of GLUT4, the major insulin-dependent glucose transporter, in primary adipocytes from women with PCOS, was found to be correlated with the women insulin sensitivity. Using miRNA databases with target gene prediction softwares, miR-93 was found to potentially target GLUT4. Over expression and inhibition of miR-93 in human adipose cells showed inverse expression of GLUT4 mRNA and protein levels [69].

Recently we have performed a large-scale miRNA expression analysis of granulosa cells isolated from PCOS patients (Imbar and Eisenberg, unpublished data). miRNAs 483-3p, 363-3p, 4284 and 212 were identified as significantly dysregulated in women with ovulation dysfunction. Bioinformatics analysis suggests an inhibitory role of miRNAs 483-3p; 363-3p; 4284 in several steps of the TGFβ signaling

Table 18.1 Dysregulated miRNAs in cumulus granulosa cells from anovulatory women with PCOS[a]

miRNA	Expression	Previous validated targets[b]
miR-423-3p	↑	CDKN1A
miR-3651	↑	
miR-3653	↑	
miR-151b	↑	
miR-1273g-3p	↑	
miR-590-5p	↑	TGFBR2
miR-3648	↑	FUT11
miR-7845-5p	↑	
miR-27a-5p	↑	
miR-1275	↑	
miR-483-3p	↑	SMAD4, BBC3,PARD3, NUDC
miR-7-5p	↑	SNCA
miR-483-5p	↑	SRF, MAPK3
miR-3529-3p	↓	
miR-7974	↓	
miR-3065-5p	↓	RPL7L1
miR-214-3p	↓	EZH2, PTEN, DAPK1, MAP2K3, MAPK8, POU4F2, PLXNB1, SRGAP1, GALNT7
miR-200a-3p	↓	DLX5, BAP1, G3BP2, PCDH8, RAB30, ZEB2, CTNNB1, ZEB1, SIP1, ZFPM2
miR-203a	↓	UVRAG, ABL1, TP63, ABCE1, CDK6, SOCS3, BCL2L2, BMI1, ZEB2,EYA4, EDNRA
miR-4732-5p	↓	
miR-423-5p	↓	RABAC1, NMD3, YWHAZ, RGPD2, TSPYL6, HIST2H2BE, USP6, PGAM4, IGDCC4
let-7a-3p	↓	ARMC8, SETD4, BTF3, MIPOL1, TRIM33
miR-3184-5p	↓	
miR-548n	↓	
miR-221-3p	↓	CDKN1B, BCL2L11, BMF, FOXO3, KIT, CDKN1C, TMED7, DDIT4, BNIP3L, TBK1
miR-149-5p	↓	SP1, FOXM1
miR-1298-5p	↓	
miR-193a-3p	↓	MCL1, E2F6, PTK2, PRAP1,
miR-365a-3p	↓	CCND1, BCL2, IL6
miR-219a-1-3p	↓	

[a]Reference [65]. Only miRNAs with expression change >3 are listed
[b]Validated target records are based on the miRTarBase

Table 18.2 Dysregulated miRNAs in follicular fluid or serum from anovulatory women with PCOS

miRNA	Tissue/Cells	Expression	Platform	Reference
miR-21	Serum	↑	TaqMan	[212][a]
miR-27b	Serum	↑	TaqMan	[212]
miR-103	Serum	↑	TaqMan	[212]
miR-155	Serum	↑	TaqMan	[212]
miR-222	Serum	↑	TLDA	[94][b]
miR-146a	Serum	↑	TLDA	[94]
miR-30c	Serum	↑	TLDA	[94]
let-7i-3pm	Serum	↑	LNA array	[213][c]
miR-5706	Serum	↑	LNA array	[213]
miR-4463	Serum	↑	LNA array	[213]
miR-3665	Serum	↑	LNA array	[213]
miR-638	Serum	↑	LNA array	[213]
miR-124-3p	Serum	↓	LNA array	[213]
miR-128	Serum	↓	LNA array	[213]
miR-29a-3p	Serum	↓	LNA array	[213]
let -7c	Serum	↓	LNA array	[213]
miR-132	Follicular fluid	↓	TLDA	[64]
miR-320	Follicular fluid	↓	TLDA	[64]
miR-32	Follicular fluid	↑	TLDA	[63]
miR-34c	Follicular fluid	↑	TLDA	[63]
miR-135a	Follicular fluid	↑	TLDA	[63]
miR-18b	Follicular fluid	↑	TLDA	[63]
miR-9	Follicular fluid	↑	TLDA	[63]

[a]Obese PCOS patients
[b]*TLDA* TaqMan low density expression array
[c]*LNA array* miRCURY locked nucleic acid expression array

pathway through alteration in the Smad3 (SMAD family member 3) and Smad4 (SMAD family member 4) expression. Previous reports demonstrated that miRNA-24 decreases TGFβ signaling by repressing the expression of Smad proteins [70]. TGFβ signaling is known to promote estradiol release [71]. Therefore, miRNA-24 regulation of estradiol concentration could be explained by a mechanism in which overexpression of miRNA-24 will decrease TGFβ signaling, which in turn will inhibit estradiol secretion.

An abundance of scientific research has demonstrated that the TGF-β family of growth factors has wide-ranging roles in female reproduction. Although various family members are expressed by the major ovarian cell types, many of their functional effects center at the control of granulosa cell growth and differentiation, both of which affect folliculogenesis and oocyte development. Disruption in the TGF-β family in mouse models had disrupted the ovarian function and resulted in a phenotype that mimics premature ovarian failure (POF) [72].

POF is defined as the lost ovarian functions before 40 years of age [73]. POF results in amenorrhea, hypoestrogenism and elevated gonadotropins, and is a definite cause of infertility [74]. There is some data relating to altered miRNA expression in POF patients usually through miRNA microarray analysis [75]. Profiling of differentially expressed miRNAs in POF provided a novel insight into the molecular events involving the role of miRNAs in POF development. Ten miRNAs showed increased expression in POF: miR-202, miR-146a, miR-125b-2*, miR-139-3p, miR-654-5p, miR-27a, miR-765, miR-23a, miR-342-3p, miR-126 and two showed decreased expression (let-7c and miR-144). None of the miRNAs associated with POF were also displayed differential expression in the studies of miRNAs expression in PCOS patients.

To understand the molecular mechanism responsible for POF development several studies analyzed individual miRNA and candidate targets using animal models [75, 76]. A total of 63 miRNAs were up-regulated and 20 miRNAs were down-regulated in the rat POF model. miRNA-29a and miRNA-144 may target expression of PLA2G4A (phospholipase A2 gene) that is involved in prostaglandin biosynthesis and is significantly up-regulated the ovaries from POF mice. Previously, it was shown that miR-133b is involved in FSH induced estrogen secretion [40]. The forkhead box L2 gene (Foxl2) transcribes to a nuclear transcription factor which is a key modulator of steroidogenesis and was showed to be involved in POF [77]. This transcription factor expression is inhibited by miR-133b [78]. miR-133b inhibits Foxl2 expression in human and mouse granulosa cells by binding to the Foxl2 mRNA. The inhibition of Foxl2 transcription impairs the negative Foxl2 mediated transcriptional regulation of StAR (steroidogenic acute regulatory protein) and CYP19A1 genes, thus stimulating estrogen production.

This study reveals the crosstalk between miR-133b, Foxl2 and estrogen synthesis, and improves our understanding of the significant roles played by miRNAs in follicular growth and ovarian function. Different study found miR-23a, also significantly upregulated in the plasma of POF patients, to be essential for apoptosis induction in hGC by targeting XIAP (X-linked inhibitor of apoptosis) and the caspase signaling cascade [75]. These results provide clear evidence, for the first time, that miR-23a promotes apoptosis of granulosa cells via decreasing XIAP expression, which may contribute to the etiology of POF.

Newborn ovary homeobox gene (NOBOX) transcribes to another distinct transcription factor. This gene is expressed in oocytes throughout folliculogenesis [79]. The importance of this gene is evident as NOBOX knockout mice are infertile, and mutations in the NOBOX gene have been associated with POF phenotype, suggesting the essential role of NOBOX in folliculogenesis [80]. Recently, a predicted miRNA recognition element (MRE) for miR-196a which is critical for the specific binding of miR-196a to the NOBOX mRNA has been found [81].

All of these identified miRNAs targets involved in ovarian dysfunction carry the potential to provide new mechanistic and therapeutic targets for clinical trials in female infertility disorders such as anovulation and POF.

miRNA Signatures as Biomarkers of Reproductive Disorders

Recent identification of miRNAs molecules as stable in body fluids [82–84] paved the way for their use as novel biomarkers amenable to clinical diagnosis in translational medicine. miRNAs represent a new class of biomarkers that can complement existing conventional markers, including metabolites, antigens and mRNA transcripts. The unique feature of miRNAs, to remain largely intact in clinical samples, highlights their potential in molecular phenotyping of reproductive disorders as well as to assess and monitor the physiological state of the ovulation process. miRNAs in serum/plasma hold great promise as minimally invasive diagnostic biomarkers offering more sensitive and specific tests than those currently available. miRNAs detected in biofluids may have a cellular or an extracellular origin.

Circulating or extracellular miRNAs have been shown to be stable and protected from RNase degradation by inclusion in various protein complexes or membranous particles such as exosomes and microvesicles [82–84]. It has also been shown that miRNA containing subcellular vesicles can be taken up by cells and cause changes in cellular gene expression during pathological conditions [85]. These findings suggest that extracellular miRNA may have biological functions akin to that of signaling molecules and hormones.

Since the discovery of extracellular miRNA and their stability in different biofluids, including serum and follicular fluid [64, 86], it is becoming evident that these small regulating RNAs can be used as informative biomarkers for the ovulation process. Attention should be focused in the clinical use of plasma/serum miRNAs.

It has been only a few years since the first studies of miRNAs as blood-based biomarkers for cancer were published [87, 88]. Since then, plasma/serum miRNA expression profiling had been found to vary significantly with changed physiological or pathological condition, such as pregnancy, heart failure and sepsis [89–91]. In the ovary, recent study is showing the use of plasma/serum miRNAs as a non-invasive marker of ovarian cancer [92].

miRNAs were identified as circulating miRNAs in the follicular fluid and in the serum of women with anovulation. The expression of miRNAs in serum of patients with PCOS had been evaluated using miRNA arrays [93, 94]. Five circulating miRNAs were found as significantly expressed in PCOS patients (let 7i-3pm; miR-5706; Mir-4463; miR 3665; Mir-638) and four (miR-124-3p; miR-128; miR-29a-3p; let-7c) had decreased expression (Table 18.2). The array study showed a different set of miRNAs: miR-222, miR-146a and miR-30c to be significantly increased in the PCOS patients. Sensitivity and specificity analysis, using receiver operating characteristic (ROC) curves and area under the curve (AUC), revealed that a combination of the three miRNAs was able to distinguish between the PCOS and controls [94]. Interestingly, elevated circulating miR-222 levels were also associated with type 2 diabetes [95].

Decreased miR-146 has been linked to inflammation and insulin resistance in Type II Diabetes patients [96]. This microRNA was negatively correlated with serum testosterone levels in PCOS women [94]. The authors conclude that although serum miRNAs are a promising biomarkers for ovarian function, most of

the miRNAs present and differentially expressed in ovarian tissue from PCOS women are not released into the blood, therefore, were not detected in the serum.

Ovarian specific miRNAs detected in the serum are expected to increase the yield of early diagnosis and have a great potential in individualized treatment. This new approach has the potential to revolutionize present clinical management, including determining ovarian reserve, ovarian endocrine function and evaluate the efficacy of ovulation inducing drugs.

microRNAs and Spermatogenesis

Spermatogenesis

Male germ cell differentiation, spermatogenesis, takes place in the epithelium of highly convoluted seminiferous tubules inside the testis [97]. Spermatogenesis is a complex differentiation program that includes three phases: (1) mitotic proliferation, (2) meiosis and (3) haploid differentiation (spermiogenesis). Spermatogonial stem cells (SSCs) located at the basal compartment of the seminiferous epithelium are able to self-renew and therefore, provide a constant pool of undifferentiated germ cells for the production of spermatozoa throughout the whole sexual maturity [98].

Spermatogenesis is initiated when SSCs enter the differentiation pathway. Differentiating spermatogonia proliferate mitotically to give rise to different types of spermatogonia (A-single, A-paired, A-aligned, A1, A2, A3, A4, B and Intermediate spermatogonia), and expand the colony of differentiating germ cells. After the proliferation phase, type B spermatogonia divide to form preleptotene spermatocytes and the long-lasting meiotic prophase I begins. During the meiotic prophase I that includes preleptotene, leptotene, zygotene, pachytene and diplotene phases, the homologous chromosomes pair to from the synaptonemal complex, chromosomes undergo crossing-over and homologous recombination to shuffle genetic material and finally, spermatocytes undergo a reduction division to split the sister chromosomes into two cells. Generated secondary spermatocytes divide again very quickly, and the resulting haploid round spermatids commence the dramatic cellular morphogenesis to construct sperm-specific structures, such as a flagellum and an acrosome, and to reshape the nucleus and compact the chromatin with the help of sperm-specific protamines that replace most of the histones. Spermatozoa that are released into the tubular lumen in a process called spermiation and continue their journey to the epididymis for final maturation and storage.

The seminiferous epithelium is organized into cyclic stages so that each cross section of the tubule contains a defined grouping of germ cell types at particular phases of development [99]. This organization enables the coordinated regulation of germ cells at different phases of differentiation by the surrounding Sertoli cells that embed germ cells in the cytoplasmic pockets and provide them with physical and nutritional support [100]. Spermatogenesis is dependent on the synchronized action

of the hypothalamic-pituitary-gonadal (HPG) hormonal axis. GnRH from the hypothalamus regulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland [101] that target somatic testicular cell types to stimulate testosterone production by interstitial Leydig cells or to regulate Sertoli cells.

The progress of spermatogenesis is accompanied by orchestrated transcriptional waves that generate specific transcriptome profiles for SSCs, differentiating mitotic spermatogonia, meiotic spermatocytes and haploid spermatids [102, 103]. In addition to protein-coding mRNAs, ncRNAs are expressed during spermatogenesis in a phase-specific manner [103–106]. In elongating spermatids, transcription ceases because of the histone-protamine transition and tight chromatin compaction. Therefore, mRNAs for many spermiogenic proteins are synthesized already in earlier stages and are temporarily stored until needed during later steps [107, 108].

Male germ cells are transcriptionally highly active and the testis has been shown to have the most complex transcriptome of all tissues, which originates mainly from spermatocytes and round spermatids. These cells express not only a diverse set of protein-coding mRNAs and isoforms but also an exceptional amount of different ncRNAs that are largely derived from uncharacterized intergenic regions [109]. Therefore, it is clear that the accurate and efficient posttranscriptional gene control is of fundamental importance for normal progression of spermatogenesis. There is a wide range of different RNA-binding proteins, including testis-specific ones, that are expressed in meiotic and post-meiotic cells to participate in mRNA regulation by recognizing target mRNAs and forming ribonucleoprotein (RNP) complexes [110, 111]. Germ cell-specific RNP granules, commonly called germ granules, accumulate RNA and RNA regulatory proteins and coordinate RNA regulation in the male germ line [112–114]. Among other posttranscriptional mechanism, miRNAs have turned out to be important regulators of gene expression in male germ cells.

Expressions of miRNAs During Spermatogenesis

Presently, 20 years after their discovery [115], miRNAs are recognized as vital regulatory factors that control the expression of a broad range of protein-coding genes [116, 117].

Several miRNAs are highly, exclusively or preferentially expressed in the testis and in specific testicular cell types as revealed by miRNA microarrays, RT-PCR or small RNA sequencing studies [118, 119]. Differentially expressed miRNAs in embryonic germ cells or SSCs have been assessed by comparing the miRNA profiles of immature and mature mouse and primate testes [120, 121], enriched populations of gonocytes and spermatogonia in the mouse [122], the Thy1(+) SSC-enriched testis cell population and the Thy1(−) cell population, composed primarily of testis somatic cells [123], and GFRa-positive spermatogonia and GFRa-negative non-stem cell spermatogonia [124]. Differential miRNA expression during post-natal spermatogenesis has been identified from mouse testis samples collected during the

first wave of spermatogenesis [125, 126] and from enriched populations of stem cells, pre-meiotic, meiotic and post-meiotic cells [127–129]. It is important to correlate the miRNA expression changes during spermatogenesis to the global expression changes of protein-coding mRNAs to build a better understanding of the role of miRNA mediated gene regulation in the testis. This kind of approach has already been initiated by a study that integrated the miRNA and mRNA profiles during the first wave of spermatogenesis into miRNA-dependent regulatory networks [130].

In addition to the germ cells, the miRNA profiles of the somatic compartment of the testis, particularly immature Sertoli cells have been assessed [131–133]. Interestingly, the expression of several miRNAs in Sertoli cells was demonstrated to be regulated by androgens [134, 135]. Many of these miRNAs are preferentially expressed in the testis and regulate genes that are highly expressed in Sertoli cells. Therefore, androgens appear to control spermatogenic events through miRNA-dependent mechanisms.

While some miRNAs are ubiquitously expressed in different cell types, a high number of miRNAs follow phase-specific expression patterns, being predominantly or specifically expressed in different somatic cell types or in germ cells at distinct phases of differentiation.

The densities of miRNA genes are higher on the mammalian X chromosome than on autosomes; this is a consequence of the organization of X-chromosomal miRNA genes in clusters that consist of paralogous copies resulting from miRNA gene duplication [136]. Comparison of miRNA levels in spermatocytes and spermatids to those in somatic cells revealed that the multimember family of miRNAs on the X chromosome have higher expression levels than all other miRNA categories in male germ cells [136]. Sex chromosomal genes undergo epigenetic silencing in spermatocytes during meiotic prophase I by meiotic sex chromosome inactivation (MSCI). However, it appears that miRNAs, particularly the miRNA families that expanded by gene duplication, escape silencing by MSCI [137]. This suggests that the miRNA gene duplications on the X chromosome were selectively favored during evolution to allow their expression in spermatocytes and spermatids despite sex chromosome inactivation.

Male germ cells express also endogenous small interfering RNAs (endo-siRNAs) that are processed from long dsRNA precursors by Dicer in a microprocessor complex (Drosha-DGCR8)-independent manner [138]. Interestingly, the majority of identified endo-siRNAs mapped to several (even hundreds of) different sites on multiple chromosomes, in contrast to miRNAs that are usually derived from a unique locus or very few loci. Target prediction suggested that the testicular endo-siRNAs mainly target mRNAs (~92 %) but also transcripts of pseudogenes (~3 %), retrotransposons (~1 %), and ncRNAs (~4 %). The function of testicular endo-siRNAs remains obscure. They can effectively induce target mRNA degradation *in vitro*, suggesting possible involvement in posttranscriptional regulation. Alternatively, endo-siRNAs could also have nuclear effects on chromatin modifications similar to what has been shown for endo-siRNAs in other organisms, as supported by their origin from multiple chromosomal loci [138].

Regulation of Spermatogonial Stem Cell Self-Renewal and Differentiation

The development of the testis begins when the Y-chromosomal testis-determining gene Sry is transiently expressed in the supporting cell precursors of the genital ridge from 10.5 to 12.5 days post coitum (dpc) in mice and triggers the chain of events leading to the differentiation of testis-specific cell types [139]. Recent studies have revealed the expression of miRNAs during the embryonic development of the testis and have demonstrated that miRNA-mediated gene control is involved in the regulation of this developmental process [140, 141]. The testis cords are formed by Sertoli cells that assemble around clusters of primordial germ cells (PGCs) that become gonocytes and peritubular cells that surround the cords [142]. Gonocytes show a burst of mitotic activity and then arrest in the G_0 phase of the cell cycle, remaining mitotically quiescent until after birth, when they become spermatogonia [143].

SSCs in the basal compartment of the seminiferous epithelium undergo self-renewal divisions to maintain the stem cell pool. Specific signals trigger the differentiation program to produce spermatozoa from spermatogonia. The stem cell status of SSCs is strictly regulated; however, little is known about the mechanisms of the acquirement and maintenance of the self-renewal activity [98]. miRNAs contribute to the regulation of SSCs. High-throughput sequencing identified that miR-21, along with miR-34c, -182, -183, and -146a, are preferentially expressed in SSC-enriched population. The downregulation of miR-21 in SSC-enriched germ cell cultures increased apoptosis and reduced the SSC potency, suggesting that miR-21 has a role in maintaining the SSC population [123]. miRNA-20 and miRNA-106a are also preferentially expressed in mouse SSCs, and they were shown to regulate the renewal of SSCs by targeting STAT3 and Ccnd1 [124].

Several studies have addressed the function of miRNAs in the regulation of the SSC's decision to initiate differentiation. For example, miR-146 is highly expressed in undifferentiated spermatogonia, and its expression is downregulated nearly 180-fold in differentiating spermatogonia [144]. miR-146 levels were dropped upon induction of differentiation by retinoic acid, and it was suggested that miR-146 is involved in the control of retinoid acid-induced spermatogonial differentiation in the mouse. Other miRNAs that are highly expressed in undifferentiated cells and downregulated upon retinoic acid treatment are the X chromosome-clustered miR-221 and miR-222 [145]. The impaired function of miR-221/miR-222 in mice induced differentiation and loss of stem cell capacity. The deletion of the Mir-17-92 cluster that is also preferentially expressed in undifferentiated spermatogonia results in small testes and a lowered epididymal sperm counts in mice [146]. Interestingly, the inactivation of Mir-17-92 cluster increased the transcript levels of another cluster, miR-106b-25, suggesting a functional co-operation of these two clusters.

Some miRNAs, such as let-7 family miRNAs, have an opposite expression pattern, being induced upon retinoic acid stimulated differentiation. The induction of let-7 family miRNAs was shown to be accompanied by downregulation of let-7

family targets Mycn, Ccnd1 and Colla2 [147]. The increase in let-7 family member expression in differentiating spermatogonia was suggested to be mediated by the simultaneous decrease in the levels of an RNA-binding protein, LIN28, which has a well-characterized role in inhibiting let-7 miRNA biogenesis [148]. Expression of LIN28 in undifferentiated spermatogonia has been confirmed by several studies [149–151], and LIN28 was shown to regulate the cyclic expansion of spermatogonial progenitor population using a knockout mouse model [152].

Regulation of Meiotic and Post-meiotic Events

Pachytene spermatocytes and round spermatids are transcriptionally active and they have exceptionally diverse transcriptomes [109]. It is therefore clear that these cells need to maintain effective posttranscriptional control. Late meiotic and haploid germ cells have also been suggested to be the main source of miRNA production during spermatogenesis [125]. The important role of miRNA-mediated regulation during meiotic and post-meiotic development is supported by the phenotypes of the male germ cell-specific *Dicer1* knockout mice that show the most dramatic spermatogenic defects during these specific phases.

miR-34c that has been shown to be involved in the regulation of stem cell status [123, 153, 154], is also an important regulator of the later steps of spermatogenesis. Several studies have revealed that miR-34c expression is highly upregulated in spermatocytes and round spermatids [155–157]. Downregulation of miR-34c expression in primary spermatocytes seems to prevent germ cells from testosterone deprivation-induced apoptosis; in line with this, overexpression of miR-34c in cultured germ cells triggers apoptosis, a process that is at least partially mediated by targeting ATF1 via direct binding to the ATF1 3′UTR. Ectopically expressed miR-34c in HeLa cells induced a shift in the transcriptome toward the germ lineage transcriptome [155]. Therefore, miR-34c was suggested to play a role in enhancing the germinal phenotype of cells already committed to this lineage. miR-449 cluster miRNAs function also in the regulation of meiotic and post-meiotic gene expression; they are drastically up-regulated at meiotic initiation and their expression is under the regulation of the transcription factors CREMtau and SOX5 [158].

Despite a high expression level in male germ cells, miR-449-null male mice exhibit normal spermatogenesis. Interestingly, miR-34b/c levels were upregulated in miR-449 knockout testis, and miR-449 cluster and miR-34b/c were shown to share some target genes that belong to the E2F transcription factor-retinoblastoma regulatory network, indicating functional redundancy [158]. The deletion of both miR-34b/c and miR-499a/b/c clusters in the mouse genome resulted in oligoasthenoteratozoospermia (OTA) and infertility, which further supports their functional redundancy, and also signifies their importance for normal spermatogenesis [159–161].

During the late steps of spermatid differentiation, the chromatin is compacted by the replacement of histones with protamines, and the process is dependent on the

correct timing of the expression of transition proteins (TP) and protamines (Prm) [162]. miRNAs are also participating in this accurately timed expression of TPs and Prms. miR-469 can target TP2 and Prm2 mRNAs and repress TP2 and Prm2 protein expression in pachytene spermatocytes and round spermatids at the translational level with a minor effect on mRNA degradation [163]. Another miRNA that controls TP2 expression is miR-122a that is enriched in late-stage male germ cells; miR-122a can induce TP2 mRNA cleavage [164]. The Oncomir-1 (miR-17-92) cluster miRNA miR-18 can directly target heat shock factor 2 (HSF2) during spermatogenesis [165]. HSF2 is a transcription factor that influences a wide range of developmental processes, including spermatogenesis [166]. The expression of HSF2 and miR-18 exhibits an inverse correlation during spermatogenesis, and downregulation of miR-18 expression in seminiferous tubules leads to increased HSF2 protein levels and altered expression of HSF2 target genes [165].

miRNA-Processing Enzymes in Spermatogenesis

miRNA and siRNA processing is dependent on two endonucleases, the microprocessor complex (Drosha/DGCR8) and Dicer, which are both essential genes for mouse development [5, 167]. The tissue and cell type-specific knockout mouse models have been helpful in understanding the role of these pathways in certain tissues and in the adult organism. The same approach has been used to study the importance of miRNA/siRNA pathways in spermatogenesis. Based on these studies, Dicer is required for male fertility, and the functions of both testicular somatic cells and germ cells are dependent on Dicer.

The deletion of *Dicer1* in the somatic cells of fetal testes with *Sf1* promoter-driven Cre showed no abnormality during fetal life, although *Dicer1* deletion was induced already in the adrenogonadal primordium [168]. However, the testis was degenerated shortly after birth; at PND5, most of the testis cords were degenerated and the number of proliferating Sertoli cells and HSD3b-postitive Leydig cells in the interstitium were decreased. The ablation of Dicer in Sertoli cells led to disrupted spermatogenesis and progressive testicular degeneration [131, 169]. The defects appeared already in the prepubertal testis due to defective Sertoli cell maturation and function, with major alterations in both the transcriptome and proteome in the neonatal testis of Sertoli cell-specific *Dicer1* knockout mice [131].

The consequences of *Dicer1* deletion in embryonic or differentiating male germ cells have been studied using conditional knockout mouse models with distinct cell type-specific promoters to induce Cre expression. Removal of *Dicer1* in (PGCs) after embryonic day 10 (E10) using tissue-nonspecific alkaline phosphatase (*Tnap*) promoter-driven Cre expression caused defects in PGC proliferation as well as postnatal spermatogenesis and fertility [170, 171]. The low penetrance of the *TnapCre* transgene hindered the exact analysis of the defects using this mouse model as a tool. *Dicer1* ablation in the male germ line just before birth using *Ddx4* promoter-driven Cre expression resulted in accumulating defects in meiotic and post-meiotic

germ cells and male infertility [156, 172]. The progression of meiotic prophase I was delayed, and the number of apoptotic spermatocytes was increased, leading to a decreased number of haploid post-meiotic round spermatids. Furthermore, the differentiation of the remaining round spermatids failed and only very few morphologically abnormal non-functional spermatozoa were produced [156].

The deletion of *Dicer1* in post-natal spermatogonia by Neurogenin3 (*Ngn3*)-*Cre* and *Stra8Cre* transgenes resulted in male infertility but the spermatogenic phenotype was somewhat less severe than the *Ddx4Cre*-driven phenotype [173–175]. Selective removal of *Dicer1* in type A spermatogonia by *Ngn3Cre* did not cause obvious problems in meiosis, and the first clear defects were manifested in haploid male germ cells. The elongation phase of spermiogenesis was severely compromised with problems in chromatin organization and sperm head shaping and compaction. Similar to the *Ddx4Cre-Dicer1* knockout model, the morphology of epididymal spermatozoa was abnormal with small misshapen heads and defective tail structures, and the number of produced spermatozoa was greatly reduced [173]. The *Stra8Cre* transgene inducing *Dicer1* deletion in differentiating spermatogonia resulted in the comparable phenotype to *Ngn3Cre*-driven *Dicer1* deletion with affected haploid differentiation [174, 175]; meiotic progression was also somewhat delayed in *Stra8Cre-Dicer1* knockout spermatocytes. Even later deletion of *Dicer1* in haploid cells using the *Protamine 1* (*Prm1*)-*Cre* transgene caused less problems in spermatogenesis, suggesting that the spermatogenic phenotype of the *Ngn3Cre* and *Sra8Cre* models originate already in the earlier pre-meiotic/meiotic cell types [176]. The *Prm1Cre-Dicer1* knockout testis had elongating spermatids with abnormal head morphology and compromised chromatin integrity. This study revealed that *Dicer1* inactivation in round spermatids impaired translational activation of germ cell transcripts, including Prm1, possibly resulting from the transcript sequestration into translationally inert RNP complexes [176].

Altogether the male germ cell-specific *Dicer1* knockout models suggest that Dicer activities are required throughout male germ cell differentiation, and earlier deletion results in the accumulation of defects and more compromised spermatogenesis. In addition, Dicer activity during the time of birth seems to be important for normal meiotic progression because this phase is dramatically impaired in the *Ddx4Cre* model compared with the less affected meiotic progression in *Ngn3Cre* or *Sra8Cre* models.

The importance of miRNA-dependent pathways in spermatogenesis has also been dissected by using knockout mouse models with defective microprocessor components (Drosha and DGRC8). *Drosha* knockout mouse model was generated using the *Stra8Cre* transgene, and the phenotype was compared with the *Stra8Cre-Dicer1* knockout [175]. Drosha has been reported to have some miRNA-independent roles, for example, in mRNA cleavage and ribosomal RNA biogenesis [177]; however, in small RNA processing, the function of Drosha is limited to the processing of miRNAs, whereas Dicer processes both miRNAs and endo-siRNAs. The comparison of *Drosha* and *Dicer1* knockout testes revealed that *Drosha* deletion caused more severe spermatogenic disruptions [175]. An opposite observation was done using *Ddx4Cre* transgene to delete *Dicer1* or *Dgcr8* gene in male germ cells.

Dgcr8 knockout male mice were infertile and displayed similar defects to *Dicer1* knockout, however the defects were less severe [178]. Therefore, the deletion of different components of the microprocessor complex (Drosha vs. DGCR8) results in somewhat different phenotypes. Because of the contradictions, no clear conclusion can be made about the relative roles of miRNA and endo-siRNA pathways in spermatogenesis. However, if the function of DGCR8 is mostly associated with microprocessor complex whereas Drosha has miRNA-independent functions, the more severe phenotype caused by the Drosha ablation could be explained by the more diverse functions of Drosha. If this is the case, the comparison of the testicular phenotypes of *Ddx4Cre*-driven *Dicer1* and *Dgcr8* knockout mice suggest that all the defects in the *Dicer1* knockout spermatogenesis cannot be explained because of the defective miRNA pathway, but other pathways (e.g. endo-siRNA pathways and small RNA-independent functions of Dicer) are also affected.

miRNAs are known to affect various cellular transcripts and therefore, several studies have been designed to understand the changes in the transcriptome in the absence of miRNA processing enzymes. The microarray analysis of the *Stra8Cre-Dicer1* knockout testis (PND18) [174] or pachytene spermatocytes and round spermatids [175] demonstrated that a high number of genes were differentially expressed in the absence of Dicer. Disproportionately higher percentage of X- and Y-linked genes was overexpressed in the PND18 *Stra8Cre-Dicer1* knockout testis, which suggests that these sex chromosomal genes could escape MSCI in *Dicer1* knockout spermatocytes. However, it remains unclear whether overexpression of the genes that should be silenced during MSCI is induced by direct defects in MSCI or whether it originates from the increased transcript stability or from the enrichment of pre-MSCI spermatocytes in the analyzed knockout testis samples. It has to be also noted that the ablation of Dicer or Drosha in spermatogonia did not significantly affect the sex body formation in pachytene spermatocytes at microscopical level. RNA sequencing of spermatocytes of *Ddx4Cre-Dicer1* mice revealed a general upregulation of gene expression [178]. Interestingly, genes related to spermatogenesis or meiosis were not found to be affected but instead, the expression of numerous lowly expressed genes were upregulated. Therefore, Dicer inactivation appears to affect the fine-tuning of protein-coding gene expression by increasing low level gene expression.

It is clear that the disruption of miRNA/endo-siRNA pathways has dramatic consequences on spermatogenesis and it is difficult to tell which ones of the affected mRNAs are direct targets of small RNAs and which mRNA expression changes are secondary effects due to the disrupted cellular processes. Furthermore, miRNA-independent functions of Dicer and Drosha further complicate the analysis of the knockout phenotypes at transcriptome level. Not only protein-coding mRNAs but also other cellular transcripts are affected in the absence of Dicer. For example, in *Ddx4Cre-Dicer1* knockout spermatocytes, the expression of transposable elements of the SINE (short interspersed nuclear element) family was up-regulated [156]. Similar up-regulation of transposon expression (MT and SINE families) was observed in *Dicer1*-deficient mouse oocytes [26]. Dicer also functions in the processing and detoxification of transposon-derived RNAs in retinal pigmented epithelium, where it

is involved in retrotransposon transcript degradation by a miRNA-independent mechanism [179]. Interestingly, transposon expression was unaffected in the *Ngn3Cre-Dicer1* knockout testis, suggesting that Dicer may be involved in transposon control in embryonic and/or very early post-natal phases before the onset of Ngn3 expression. On the other hand, major satellite expression was induced in the testis of *Ngn3Cre-Dicer1* knockout mice, similar to what is observed in mouse embryonic stem cells lacking functional Dicer [173, 180, 181]. The diverse function of Dicer in both miRNA-dependent gene regulation and miRNA-independent processes highlight the need for different mouse models that target specific pathways to understand the exact roles of miRNAs during spermatogenesis.

miRNAs and Human Male Fertility

Spermatogenesis is a complex process that is also error-prone. Indeed, human semen samples are considered normal with only 4 % of morphologically normal spermatozoa [182]. Moreover, alarming adverse trends in semen quality and the incidence of testicular germ cell tumors and congenital malformations of the reproductive organs in western countries have been reported [183], raising concern about environmental and genetic factors that negatively affect male reproductive health. Increasing infertility is reflected by an increased use of assisted reproductive technologies (ART). Paternal factors are involved in the etiology of infertility or subfertility in approximately half of the cases undergoing ART [184]. Reliable diagnosis tools are available for some obvious forms of male infertility with clear changes in observable semen parameters; however, sometimes the external characteristics of the sample may appear normal but still the fertility of sperm is compromised. Therefore, additional biomarkers to evaluate the quality of the sperm sample and sperm fertility status are needed. Furthermore, there has been speculation concerning the phenotype and health of the offspring produced from IVF and ICSI [185, 186], further highlighting the requirement to better understand the etiology of male infertility prior to ART.

It is important to understand the gene expression changes during spermatogenesis to correlate the possible changes in the transcriptome with specific types of male infertility. The human testicular transcriptome has been analyzed using testis biopsies from prepubertal children and adult patients with arrested spermatogenesis at different stages to gain insight into the transcriptome of different populations of testicular cells in humans [187]. Considering the crucial role of miRNAs for the normal progress of mouse spermatogenesis, it is reasonable to assume that miRNAs may play a role in the etiology of human male subfertility and infertility. miRNA profiles of human spermatogonia, pachytene spermatocytes and rounds spermatids were recently identified by miRNA microarrays, and several differentially expressed miRNAs were identified [188]. To understand the role of miRNAs in the human spermatogenic failure, miRNA expression profiles have been studied in normal testicular tissues or in the testes of men with Sertoli cell only (SCO), mixed atrophy (MA) and germ cell arrest (GA) histopathologic patterns.

Definite analysis of testicular samples is challenging due to the complex organization of spermatogenic cells in the seminiferous epithelium and the heterogeneity of the testicular samples. Therefore, spermatozoal RNA isolated from semen samples appears to be a more feasible for diagnostic purpose than invasive testicular biopsies. Despite the transcriptionally inactive state of mature spermatozoa, RNA profiling experiments have identified a complex population of RNAs in mature spermatozoa both in human and other species [189–191]. Spermatozoal RNAs include rRNA, mRNA and both long and small ncRNAs. Among other small ncRNAs, such as piRNAs and small RNAs originating from repetitive elements or transcription start sites/promoters, miRNAs have also been identified in human spermatozoa [192, 193]. The functional importance of spermatozoal RNAs has remained unclear. Many transcripts in spermatozoa appear compromised, and it is possible that some of them are remnants of functional RNAs from earlier developmental stages [109, 191]. However, at least some of the sperm-specific transcripts are transmitted to the oocyte upon fertilization and potentially provide a post-fertilization function in early embryonic development [194] or epigenetic inheritance [195].

Spermatozoal miRNAs have been suggested to act during early embryogenesis by controlling the early embryonic gene expression [196]. miR-34c, which is the most abundant sperm miRNA in humans [192], is also found in mature sperm and zygotes in mice, and the injection of the miR-34c inhibitor into zygotes was reported to attenuate the first cleavage division after fertilization [197]. However, a recent study demonstrated that the presence of miR-34b/c and miR-449a/b/c cluster miRNAs in sperm is dispensable for fertilization and preimplantation development *in vivo* [161]. Therefore, direct evidence on the function of sperm-borne miRNAs in the regulation of post-fertilization events is still missing.

Many studies have been conducted to search for differentially expressed spermatozoal protein-coding mRNAs in cases of asthenozoospermia, teratozoospermia, oligozoospermia and idiopathic infertility [190, 196, 198, 199]. Similar experiments have been carried out to identify the miRNA expression profiles from spermatozoa of patients with different spermatogenic impairments (e.g., asthenozoospermic and oligoasthenozoospermic males) [200, 201]. In addition to the spermatozoal miRNAs, cell-free seminal miRNAs that are predominately derived from the testis and epididymis have been identified [202–204]. For example, three miRNAs, miR-141, miR-429 and miR-7-1-3p, were shown to be significantly increased in the seminal plasma of patients with non-obstructive azoospermia compared with fertile controls [205]. Because the expression of profiles of spermatozoal and seminal plasma miRNAs appear to be altered in patients with spermatogenic defects, they may emerge as potential biomarkers for the diagnosis and classification of male infertility.

microRNAs and the Placenta

Following implantation of the nascent embryo via coordinated/directed trophectoderm-endometrial interaction, success of the pregnancy as well as health of the fetus and mother are largely dependent on appropriate differentiation of the placental trophoblast cell lineages [206].

For the placenta will play an essential role in conversion of the spiral arteries and establishment and maintenance of the placental bed. It is the placenta that will nourish the fetus during pregnancy, perform gas exchange and act as an endocrine organ. Furthermore, the unique environment of the maternal fetal interface protects the fetus from infection while allowing the hemi-allogenic placental cells to invade the maternal spiral arteries and the uterus. With an essential role in health and maintenance of pregnancy it is not surprising that the study of miRNAs in the placenta and also in the sera of pregnant women is an area of active investigation in both normal and pathological pregnancies. The subject of miRNA expression and function during pregnancy could be a chapter topic in and of itself and is beyond the scope of this chapter. Therefore we will mention three areas of active miRNA investigation in the placenta, including: trophoblast development, protection from viral infection and a role in pregnancy disorders.

Firstly, the placenta in the human is an organ with a 9-month lifespan where tissue is available for study at many of the critical stages in its development. Progenitor cytotrophoblasts maintained throughout pregnancy, differentiate into the syncytiotrophoblast or invasive trophoblast cell lineages. Consequently, specific trophoblast lineage and temporally regulated miRNAs have been studied in the human placenta and in trophoblast cell lines. As reviewed by Morales-Prieto and colleagues differences in over 100-fold expression levels were found in miRNAs up or down regulated when comparing hundreds of miRNAs expressed in first and third trimester trophoblast cells [207]. Several of these miRNAs appear to be clustered in defined chromosomal regions with implications to imprinting as well as evolutionary placental development. Some of these miRNAs can be found to be differentially regulated, have the potential to be used as biomarkers from the sera of women with pregnancy disorders as compared to normal pregnancies.

The placenta and the maternal fetal interface as a whole play an important role in preventing infection of the fetus. A recent landmark study showed that cultured human trophoblasts are highly resistant to viral infection [208]. Furthermore, trophoblasts can confer this resistance to non-placental cells via exsosome mediated transfer of specific miRNAs. Placental exosomes are thought to be extracellular vesicles of trophoblasts that can circulate in the maternal blood stream and may act to transfer trophoblast protein and RNA. Interestingly, these exsosomes transferring viral resistance properties contained miRNAs from one of the chromosomal regions (the chromosome 19 miRNA cluster) that are expressed in a trophoblast specific lineage pattern. These exosomes induced autophagy in the recipient cells and via this mechanism inhibited replication of the virus.

miRNAs have also been implicated in pregnancy disorders, including preeclampsia [209]. Preeclampsia is a hypertensive disease of pregnancy with significant maternal and fetal morbidity and mortality worldwide. It is thought to be a two stage disease with incomplete conversion of the spiral arteries leading to defective uteroplacental perfusion and placental oxidative stress. Betoni and colleagues performed a meta-analysis of several microarrays—placenta based studies, and found that there was a discrepancy for more than 75 % of the miRNAs implicated in the pathobiology of preeclampsia [210]. Much effort has been placed in identifying biomarkers in both the sera and placenta of women for the detection of preeclampsia before

the appearance of clinical signs. Most recently high throughput analysis has been used to identify gene regulatory networks incorporating miRNA expression data to study expression of specific proteins in trophoblast cells cultured in vitro under hypoxic conditions [211].

In summary, the placenta provides an opportunity to reveal the role of miRNAs in such diverse processes as cell differentiation and development, cell invasion, oxidative stress, angiogenesis, immune regulation, protection from infection and disease prediction. The field of miRNA in the placenta is still in its infancy and awaits further investigation and study.

Conclusion

This chapter took a broad look at miRNAs expression through different parts of the reproductive system. Many studies in ovarian cells succeed in uncovering miRNAs influence on fundamental ovarian features like steroidogenesis, ovulation, and corpus luteum development and function. We emphasized the central role of miRNAs in the regulation of spermatogenesis and male fertility. A central role for these molecules is also being depicted in the formation and function of the placenta as early from the implantation of a blastocyst in the receptive endometrium and till later stages of pregnancy.

A focus on their function in pathologic processes, such as anovulation and POF is believed to lead to potential applications in ovulation induction, sperm maturation, ART and pregnancy complications.

The intensive research and the above mentioned findings suggest potential important translational implications that may lead to clinical development of miRNA based diagnostics and therapeutic tools in human fertility and reproduction. As technologies continue to improve, miRNA profiling is getting easier, faster and cheaper. At the time of this chapter publication, there were more then 200 clinical trials enlisted in the NIH clinical trials web site involving miRNAs. Personalized miRNAs based therapy is already into phase I/II trials in cancer and cardiac patients. We believe that the near future will bring translational implications of this extensive miRNAs research also into the field of human reproduction.

References

1. Lau PP, Chang BH, Chan L. Two-hybrid cloning identifies an RNA-binding protein, GRY-RBP, as a component of apobec-1 editosome. Biochem Biophys Res Commun. 2001;282(4): 977–83.
2. Ambros V. The functions of animal microRNAs. Nature. 2004;431(7006):350–5.
3. Lai EC. microRNAs: runts of the genome assert themselves. Curr Biol. 2003;13(23): R925–36.

4. Plasterk RH. Micro RNAs in animal development. Cell. 2006;124(5):877–81.
5. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, et al. Dicer is essential for mouse development. Nat Genet. 2003;35(3):215–7.
6. Ro S, Song R, Park C, Zheng H, Sanders KM, Yan W. Cloning and expression profiling of small RNAs expressed in the mouse ovary. RNA. 2007;13(12):2366–80; PMCID: 2080611.
7. Huang J, Ju Z, Li Q, Hou Q, Wang C, Li J, et al. Solexa sequencing of novel and differentially expressed microRNAs in testicular and ovarian tissues in Holstein cattle. Int J Biol Sci. 2011;7(7):1016–26; PMCID: 3164151.
8. Ahn HW, Morin RD, Zhao H, Harris RA, Coarfa C, Chen ZJ, et al. MicroRNA transcriptome in the newborn mouse ovaries determined by massive parallel sequencing. Mol Hum Reprod. 2010;16(7):463–71; PMCID: 2882868.
9. Mishima T, Takizawa T, Luo SS, Ishibashi O, Kawahigashi Y, Mizuguchi Y, et al. MicroRNA (miRNA) cloning analysis reveals sex differences in miRNA expression profiles between adult mouse testis and ovary. Reproduction. 2008;136(6):811–22.
10. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell. 2007;129(7):1401–14; PMCID: 2681231.
11. McBride D, Carre W, Sontakke SD, Hogg CO, Law A, Donadeu FX, et al. Identification of miRNAs associated with the follicular-luteal transition in the ruminant ovary. Reproduction. 2012;144(2):221–33.
12. Tripurani SK, Xiao C, Salem M, Yao J. Cloning and analysis of fetal ovary microRNAs in cattle. Anim Reprod Sci. 2010;120(1-4):16–22.
13. Hossain MM, Ghanem N, Hoelker M, Rings F, Phatsara C, Tholen E, et al. Identification and characterization of miRNAs expressed in the bovine ovary. BMC Genomics. 2009;10:443; PMCID: 2762473.
14. Li M, Liu Y, Wang T, Guan J, Luo Z, Chen H, et al. Repertoire of porcine microRNAs in adult ovary and testis by deep sequencing. Int J Biol Sci. 2011;7(7):1045–55; PMCID: 3174389.
15. Hossain MM, Sohel MM, Schellander K, Tesfaye D. Characterization and importance of microRNAs in mammalian gonadal functions. Cell Tissue Res. 2012;349(3):679–90.
16. Luense LJ, Carletti MZ, Christenson LK. Role of Dicer in female fertility. Trends Endocrinol Metab. 2009;20(6):265–72; PMCID: 3121329.
17. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2): 281–97.
18. Mallory AC, Dugas DV, Bartel DP, Bartel B. MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. Curr Biol. 2004;14(12):1035–46.
19. Borchert GM, Lanier W, Davidson BL. RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol. 2006;13(12):1097–101.
20. Lee YS, Nakahara K, Pham JW, Kim K, He Z, Sontheimer EJ, et al. Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell. 2004;117(1): 69–81.
21. Carletti MZ, Christenson LK. MicroRNA in the ovary and female reproductive tract. J Anim Sci. 2009;87(14 Suppl):E29–38; PMCID: 3118666.
22. Su AI, Cooke MP, Ching KA, Hakak Y, Walker JR, Wiltshire T, et al. Large-scale analysis of the human and mouse transcriptomes. Proc Natl Acad Sci U S A. 2002;99(7):4465–70; PMCID: 123671.
23. Nicholson RH, Nicholson AW. Molecular characterization of a mouse cDNA encoding Dicer, a ribonuclease III ortholog involved in RNA interference. Mamm Genome. 2002;13(2): 67–73.
24. Watanabe T, Totoki Y, Toyoda A, Kaneda M, Kuramochi-Miyagawa S, Obata Y, et al. Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. Nature. 2008;453(7194):539–43.

25. Cui XS, Shen XH, Kim NH. Dicer1 expression in preimplantation mouse embryos: involvement of Oct3/4 transcription at the blastocyst stage. Biochem Biophys Res Commun. 2007; 352(1):231–6.
26. Murchison EP, Stein P, Xuan Z, Pan H, Zhang MQ, Schultz RM, et al. Critical roles for Dicer in the female germline. Genes Dev. 2007;21(6):682–93; PMCID: 1820942.
27. Tang F, Kaneda M, O'Carroll D, Hajkova P, Barton SC, Sun YA, et al. Maternal microRNAs are essential for mouse zygotic development. Genes Dev. 2007;21(6):644–8; PMCID: 1820938.
28. Schier AF. The maternal-zygotic transition: death and birth of RNAs. Science. 2007; 316(5823):406–7.
29. Hong X, Luense LJ, McGinnis LK, Nothnick WB, Christenson LK. Dicer1 is essential for female fertility and normal development of the female reproductive system. Endocrinology. 2008;149(12):6207–12; PMCID: 2613048.
30. Nagaraja AK, Andreu-Vieyra C, Franco HL, Ma L, Chen R, Han DY, et al. Deletion of Dicer in somatic cells of the female reproductive tract causes sterility. Mol Endocrinol. 2008;22(10):2336–52; PMCID: 2582529.
31. Lei L, Jin S, Gonzalez G, Behringer RR, Woodruff TK. The regulatory role of Dicer in folliculogenesis in mice. Mol Cell Endocrinol. 2010;315(1–2):63–73; PMCID: 2814883.
32. Mattiske DM, Han L, Mann JR. Meiotic maturation failure induced by DICER1 deficiency is derived from primary oocyte ooplasm. Reproduction. 2009;137(4):625–32.
33. Fiedler SD, Carletti MZ, Hong X, Christenson LK. Hormonal regulation of MicroRNA expression in periovulatory mouse mural granulosa cells. Biol Reprod. 2008;79(6):1030–7; PMCID: 2780477.
34. Otsuka M, Zheng M, Hayashi M, Lee JD, Yoshino O, Lin S, et al. Impaired microRNA processing causes corpus luteum insufficiency and infertility in mice. J Clin Invest. 2008;118(5):1944–54; PMCID: 2289794.
35. Gonzalez G, Behringer RR. Dicer is required for female reproductive tract development and fertility in the mouse. Mol Reprod Dev. 2009;76(7):678–88; PMCID: 2752678.
36. Pastorelli LM, Wells S, Fray M, Smith A, Hough T, Harfe BD, et al. Genetic analyses reveal a requirement for Dicer1 in the mouse urogenital tract. Mamm Genome. 2009;20(3): 140–51.
37. Sirotkin AV, Ovcharenko D, Grossmann R, Laukova M, Mlyncek M. Identification of microRNAs controlling human ovarian cell steroidogenesis via a genome-scale screen. J Cell Physiol. 2009;219(2):415–20.
38. Yao G, Yin M, Lian J, Tian H, Liu L, Li X, et al. MicroRNA-224 is involved in transforming growth factor-beta-mediated mouse granulosa cell proliferation and granulosa cell function by targeting Smad4. Mol Endocrinol. 2010;24(3):540–51.
39. Lannes J, L'Hote D, Garrel G, Laverriere JN, Cohen-Tannoudji J, Querat B. A MicroRNA-132/212 pathway mediates GnRH activation of FSH expression. Mol Endocrinol. 2015;29(3):364–72.
40. Yao N, Yang BQ, Liu Y, Tan XY, Lu CL, Yuan XH, et al. Follicle-stimulating hormone regulation of microRNA expression on progesterone production in cultured rat granulosa cells. Endocrine. 2010;38(2):158–66.
41. Christenson LK. MicroRNA control of ovarian function. Anim Reprod. 2010;7(3):129–33; PMCID: 3111027.
42. Xu S, Linher-Melville K, Yang BB, Wu D, Li J. Micro-RNA378 (miR-378) regulates ovarian estradiol production by targeting aromatase. Endocrinology. 2011;152(10):3941–51; PMCID: 3176644.
43. Toms D, Xu S, Pan B, Wu D, Li J. Progesterone receptor expression in granulosa cells is suppressed by microRNA-378-3p. Mol Cell Endocrinol. 2015;399:95–102.
44. Wang H, Graham I, Hastings R, Gunewardena S, Brinkmeier ML, Conn PM, et al. Gonadotrope-specific deletion of Dicer results in severely suppressed gonadotropins and fertility defects. J Biol Chem. 2015;290(5):2699–714; PMCID: 4317015.

45. Hasuwa H, Ueda J, Ikawa M, Okabe M. miR-200b and miR-429 function in mouse ovulation and are essential for female fertility. Science. 2013;341(6141):71–3.
46. Lamouille S, Subramanyam D, Blelloch R, Derynck R. Regulation of epithelial-mesenchymal and mesenchymal-epithelial transitions by microRNAs. Curr Opin Cell Biol. 2013;25(2):200–7; PMCID: 4240277.
47. Ma L, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature. 2007;449(7163):682–8.
48. Gillis AJ, Stoop HJ, Hersmus R, Oosterhuis JW, Sun Y, Chen C, et al. High-throughput microRNAome analysis in human germ cell tumours. J Pathol. 2007;213(3):319–28.
49. Blenkiron C, Goldstein LD, Thorne NP, Spiteri I, Chin SF, Dunning MJ, et al. MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biol. 2007;8(10):R214; PMCID: 2246288.
50. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006; 6(11):857–66.
51. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature. 2005;435(7043):834–8.
52. van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. Science. 2007;316(5824): 575–9.
53. Ono K, Kuwabara Y, Han J. MicroRNAs and cardiovascular diseases. FEBS J. 2011;278(10):1619–33; PMCID: 3087823.
54. Pfeffer S, Sewer A, Lagos-Quintana M, Sheridan R, Sander C, Grasser FA, et al. Identification of microRNAs of the herpesvirus family. Nat Methods. 2005;2(4):269–76.
55. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science. 2005;309(5740):1577–81.
56. Wei YF, Cui GY, Ye P, Chen JN, Diao HY. MicroRNAs may solve the mystery of chronic hepatitis B virus infection. World J Gastroenterol. 2013;19(30):4867–76; PMCID: 3740416.
57. Singh PK, Singh AV, Chauhan DS. Current understanding on micro RNAs and its regulation in response to Mycobacterial infections. J Biomed Sci. 2013;20:14; PMCID: 3599176.
58. Ono K. MicroRNA links obesity and impaired glucose metabolism. Cell Res. 2011;21(6):864–6; PMCID: 3203709.
59. Rao P, Benito E, Fischer A. MicroRNAs as biomarkers for CNS disease. Front Mol Neurosci. 2013;6:39; PMCID: 3840814.
60. Hitachi K, Tsuchida K. Role of microRNAs in skeletal muscle hypertrophy. Front Physiol. 2013;4:408; PMCID: 3893574.
61. Norman RJ, Dewailly D, Legro RS, Hickey TE. Polycystic ovary syndrome. Lancet. 2007;370(9588):685–97.
62. Gilling-Smith C, Willis DS, Beard RW, Franks S. Hypersecretion of androstenedione by isolated thecal cells from polycystic ovaries. J Clin Endocrinol Metab. 1994;79(4):1158–65.
63. Roth LW, McCallie B, Alvero R, Schoolcraft WB, Minjarez D, Katz-Jaffe MG. Altered microRNA and gene expression in the follicular fluid of women with polycystic ovary syndrome. J Assist Reprod Genet. 2014;31(3):355–62; PMCID: 3947080.
64. Sang Q, Yao Z, Wang H, Feng R, Wang H, Zhao X, et al. Identification of microRNAs in human follicular fluid: characterization of microRNAs that govern steroidogenesis in vitro and are associated with polycystic ovary syndrome in vivo. J Clin Endocrinol Metab. 2013;98(7):3068–79.
65. Xu B, Zhang YW, Tong XH, Liu YS. Characterization of microRNA profile in human cumulus granulosa cells: Identification of microRNAs that regulate Notch signaling and are associated with PCOS. Mol Cell Endocrinol. 2015;404:26–36.
66. Yin M, Wang X, Yao G, Lu M, Liang M, Sun Y, et al. Transactivation of micrornA-320 by microRNA-383 regulates granulosa cell functions by targeting E2F1 and SF-1 proteins. J Biol Chem. 2014;289(26):18239–57; PMCID: 4140302.

67. Burks DJ, Font de Mora J, Schubert M, Withers DJ, Myers MG, Towery HH, et al. IRS-2 pathways integrate female reproduction and energy homeostasis. Nature. 2000;407(6802): 377–82.
68. Schmidt J, Weijdegard B, Mikkelsen AL, Lindenberg S, Nilsson L, Brannstrom M. Differential expression of inflammation-related genes in the ovarian stroma and granulosa cells of PCOS women. Mol Hum Reprod. 2014;20(1):49–58.
69. Chen YH, Heneidi S, Lee JM, Layman LC, Stepp DW, Gamboa GM, et al. miRNA-93 inhibits GLUT4 and is overexpressed in adipose tissue of polycystic ovary syndrome patients and women with insulin resistance. Diabetes. 2013;62(7):2278–86; PMCID: 3712080.
70. Ye X, Hemida MG, Qiu Y, Hanson PJ, Zhang HM, Yang D. MiR-126 promotes coxsackievirus replication by mediating cross-talk of ERK1/2 and Wnt/beta-catenin signal pathways. Cell Mol Life Sci. 2013;70(23):4631–44.
71. Knight PG, Glister C. TGF-beta superfamily members and ovarian follicle development. Reproduction. 2006;132(2):191–206.
72. Persani L, Rossetti R, Cacciatore C, Fabre S. Genetic defects of ovarian TGF-beta-like factors and premature ovarian failure. J Endocrinol Invest. 2011;34(3):244–51.
73. Coulam CB, Adamson SC, Annegers JF. Incidence of premature ovarian failure. Obstet Gynecol. 1986;67(4):604–6.
74. Nelson LM. Clinical practice. Primary ovarian insufficiency. N Engl J Med. 2009;360(6):606–14; PMCID: 2762081.
75. Yang X, Zhou Y, Peng S, Wu L, Lin HY, Wang S, et al. Differentially expressed plasma microRNAs in premature ovarian failure patients and the potential regulatory function of mir-23a in granulosa cell apoptosis. Reproduction. 2012;144(2):235–44.
76. Kuang H, Han D, Xie J, Yan Y, Li J, Ge P. Profiling of differentially expressed microRNAs in premature ovarian failure in an animal model. Gynecol Endocrinol. 2014;30(1):57–61.
77. Uhlenhaut NH, Treier M. Forkhead transcription factors in ovarian function. Reproduction. 2011;142(4):489–95.
78. Dai A, Sun H, Fang T, Zhang Q, Wu S, Jiang Y, et al. MicroRNA-133b stimulates ovarian estradiol synthesis by targeting Foxl2. FEBS Lett. 2013;587(15):2474–82.
79. Zheng P, Dean J. Oocyte-specific genes affect folliculogenesis, fertilization, and early development. Semin Reprod Med. 2007;25(4):243–51.
80. Rajkovic A, Pangas SA, Ballow D, Suzumori N, Matzuk MM. NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. Science. 2004;305(5687): 1157–9.
81. Tripurani SK, Lee KB, Wee G, Smith GW, Yao J. MicroRNA-196a regulates bovine newborn ovary homeobox gene (NOBOX) expression during early embryogenesis. BMC Dev Biol. 2011;11:25; PMCID: 3103443.
82. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008; 18(10):997–1006.
83. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105(30):10513–8; PMCID: 2492472.
84. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. Clin Chem. 2010;56(11):1733–41.
85. Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL, et al. Functional delivery of viral miRNAs via exosomes. Proc Natl Acad Sci U S A. 2010;107(14):6328–33; PMCID: 2851954.
86. Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, et al. Serum microRNAs are promising novel biomarkers. PLoS One. 2008;3(9), e3148; PMCID: 2519789.
87. Resnick KE, Alder H, Hagan JP, Richardson DL, Croce CM, Cohn DE. The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform. Gynecol Oncol. 2009;112(1):55–9.

88. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6):654–9.
89. Chim SS, Shing TK, Hung EC, Leung TY, Lau TK, Chiu RW, et al. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem. 2008;54(3):482–90.
90. Marfella R, Di Filippo C, Potenza N, Sardu C, Rizzo MR, Siniscalchi M, et al. Circulating microRNA changes in heart failure patients treated with cardiac resynchronization therapy: responders vs. non-responders. Eur J Heart Fail. 2013;15(11):1277–88.
91. Wang JF, Yu ML, Yu G, Bian JJ, Deng XM, Wan XJ, et al. Serum miR-146a and miR-223 as potential new biomarkers for sepsis. Biochem Biophys Res Commun. 2010;394(1):184–8.
92. Zheng H, Liu JY, Song FJ, Chen KX. Advances in circulating microRNAs as diagnostic and prognostic markers for ovarian cancer. Cancer Biol Med. 2013;10(3):123–30; PMCID: 3860338.
93. Ding CF, Chen WQ, Zhu YT, Bo YL, Hu HM, Zheng RH. Circulating microRNAs in patients with polycystic ovary syndrome. Hum Fertil. 2015;18(1):22–9.
94. Long W, Zhao C, Ji C, Ding H, Cui Y, Guo X, et al. Characterization of serum microRNAs profile of PCOS and identification of novel non-invasive biomarkers. Cell Physiol Biochem. 2014;33(5):1304–15.
95. Ortega FJ, Mercader JM, Moreno-Navarrete JM, Rovira O, Guerra E, Esteve E, et al. Profiling of circulating microRNAs reveals common microRNAs linked to type 2 diabetes that change with insulin sensitization. Diabetes Care. 2014;37(5):1375–83.
96. Balasubramanyam M, Aravind S, Gokulakrishnan K, Prabu P, Sathishkumar C, Ranjani H, et al. Impaired miR-146a expression links subclinical inflammation and insulin resistance in Type 2 diabetes. Mol Cell Biochem. 2011;351(1–2):197–205.
97. Jan SZ, Hamer G, Repping S, de Rooij DG, van Pelt AM, Vormer TL. Molecular control of rodent spermatogenesis. Biochim Biophys Acta. 2012;1822(12):1838–50.
98. Kanatsu-Shinohara M, Shinohara T. Spermatogonial stem cell self-renewal and development. Annu Rev Cell Dev Biol. 2013;29:163–87.
99. Kotaja N, Kimmins S, Brancorsini S, Hentsch D, Vonesch JL, Davidson I, et al. Preparation, isolation and characterization of stage-specific spermatogenic cells for cellular and molecular analysis. Nat Methods. 2004;1(3):249–54.
100. Rato L, Alves MG, Socorro S, Duarte AI, Cavaco JE, Oliveira PF. Metabolic regulation is important for spermatogenesis. Nat Rev Urol. 2012;9(6):330–8.
101. Ruwanpura SM, McLachlan RI, Meachem SJ. Hormonal regulation of male germ cell development. J Endocrinol. 2010;205(2):117–31.
102. Chalmel F, Rolland AD, Niederhauser-Wiederkehr C, Chung SS, Demougin P, Gattiker A, et al. The conserved transcriptome in human and rodent male gametogenesis. Proc Natl Acad Sci U S A. 2007;104(20):8346–51; PMCID: 1864911.
103. Laiho A, Kotaja N, Gyenesei A, Sironen A. Transcriptome profiling of the murine testis during the first wave of spermatogenesis. PLoS One. 2013;8(4), e61558; PMCID: 3629203.
104. Chalmel F, Lardenois A, Evrard B, Rolland AD, Sallou O, Dumargne MC, et al. High-resolution profiling of novel transcribed regions during rat spermatogenesis. Biol Reprod. 2014;91(1):5.
105. Sun J, Lin Y, Wu J. Long non-coding RNA expression profiling of mouse testis during postnatal development. PLoS One. 2013;8(10), e75750; PMCID: 3794988.
106. Bao J, Wu J, Schuster AS, Hennig GW, Yan W. Expression profiling reveals developmentally regulated lncRNA repertoire in the mouse male germline. Biol Reprod. 2013;89(5):107; PMCID: 4076377.
107. Kimmins S, Kotaja N, Davidson I, Sassone-Corsi P. Testis-specific transcription mechanisms promoting male germ-cell differentiation. Reproduction. 2004;128(1):5–12.
108. Kimmins S, Sassone-Corsi P. Chromatin remodelling and epigenetic features of germ cells. Nature. 2005;434(7033):583–9.

109. Soumillon M, Necsulea A, Weier M, Brawand D, Zhang X, Gu H, et al. Cellular source and mechanisms of high transcriptome complexity in the mammalian testis. Cell Rep. 2013; 3(6):2179–90.
110. Paronetto MP, Sette C. Role of RNA-binding proteins in mammalian spermatogenesis. Int J Androl. 2010;33(1):2–12.
111. Idler RK, Yan W. Control of messenger RNA fate by RNA-binding proteins: an emphasis on mammalian spermatogenesis. J Androl. 2012;33(3):309–37.
112. Kotaja N, Bhattacharyya SN, Jaskiewicz L, Kimmins S, Parvinen M, Filipowicz W, et al. The chromatoid body of male germ cells: similarity with processing bodies and presence of Dicer and microRNA pathway components. Proc Natl Acad Sci U S A. 2006;103(8):2647–52; PMCID: 1413789.
113. Kotaja N, Sassone-Corsi P. The chromatoid body: a germ-cell-specific RNA-processing centre. Nat Rev Mol Cell Biol. 2007;8(1):85–90.
114. Meikar O, Da Ros M, Korhonen H, Kotaja N. Chromatoid body and small RNAs in male germ cells. Reproduction. 2011;142(2):195–209.
115. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75(5):843–54.
116. Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. Nat Rev Genet. 2009;10(2):94–108; PMCID: 2724769.
117. Ketting RF. The many faces of RNAi. Dev Cell. 2011;20(2):148–61.
118. McIver SC, Roman SD, Nixon B, McLaughlin EA. miRNA and mammalian male germ cells. Hum Reprod Update. 2012;18(1):44–59.
119. Papaioannou MD, Nef S. microRNAs in the testis: building up male fertility. J Androl. 2010;31(1):26–33.
120. Yan N, Lu Y, Sun H, Tao D, Zhang S, Liu W, et al. A microarray for microRNA profiling in mouse testis tissues. Reproduction. 2007;134(1):73–9.
121. Yan N, Lu Y, Sun H, Qiu W, Tao D, Liu Y, et al. Microarray profiling of microRNAs expressed in testis tissues of developing primates. J Assist Reprod Genet. 2009;26(4):179–86; PMCID: 2682186.
122. McIver SC, Stanger SJ, Santarelli DM, Roman SD, Nixon B, McLaughlin EA. A unique combination of male germ cell miRNAs coordinates gonocyte differentiation. PLoS One. 2012;7(4), e35553; PMCID: 3334999.
123. Niu Z, Goodyear SM, Rao S, Wu X, Tobias JW, Avarbock MR, et al. MicroRNA-21 regulates the self-renewal of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A. 2011;108(31):12740–5; PMCID: 3150879.
124. He Z, Jiang J, Kokkinaki M, Tang L, Zeng W, Gallicano I, et al. MiRNA-20 and mirna-106a regulate spermatogonial stem cell renewal at the post-transcriptional level via targeting STAT3 and Ccnd1. Stem Cells. 2013;31(10):2205–17; PMCID: 3859454.
125. Ro S, Park C, Sanders KM, McCarrey JR, Yan W. Cloning and expression profiling of testis-expressed microRNAs. Dev Biol. 2007;311(2):592–602; PMCID: 2121622.
126. Buchold GM, Coarfa C, Kim J, Milosavljevic A, Gunaratne PH, Matzuk MM. Analysis of microRNA expression in the prepubertal testis. PLoS One. 2010;5(12), e15317; PMCID: 3012074.
127. Ro S, Park C, Young D, Sanders KM, Yan W. Tissue-dependent paired expression of miRNAs. Nucleic Acids Res. 2007;35(17):5944–53; PMCID: 2034466.
128. Marcon E, Babak T, Chua G, Hughes T, Moens PB. miRNA and piRNA localization in the male mammalian meiotic nucleus. Chromosome Res. 2008;16(2):243–60.
129. Smorag L, Zheng Y, Nolte J, Zechner U, Engel W, Pantakani DV. MicroRNA signature in various cell types of mouse spermatogenesis: evidence for stage-specifically expressed miRNA-221, -203 and -34b-5p mediated spermatogenesis regulation. Biol Cell. 2012; 104(11):677–92.
130. Sree S, Radhakrishnan K, Indu S, Kumar PG. Dramatic changes in 67 miRNAs during initiation of first wave of spermatogenesis in Mus musculus testis: global regulatory insights generated by miRNA-mRNA network analysis. Biol Reprod. 2014;91(3):69.

131. Papaioannou MD, Pitetti JL, Ro S, Park C, Aubry F, Schaad O, et al. Sertoli cell Dicer is essential for spermatogenesis in mice. Dev Biol. 2009;326(1):250–9; PMCID: 2705812.
132. Papaioannou MD, Lagarrigue M, Vejnar CE, Rolland AD, Kuhne F, Aubry F, et al. Loss of Dicer in Sertoli cells has a major impact on the testicular proteome of mice. Mol Cell Proteomics. 2011;10(4):M900587MCP200; PMCID: 3069350.
133. Ortogero N, Hennig GW, Langille C, Ro S, McCarrey JR, Yan W. Computer-assisted annotation of murine Sertoli cell small RNA transcriptome. Biol Reprod. 2013;88(1):3.
134. Nicholls PK, Harrison CA, Walton KL, McLachlan RI, O'Donnell L, Stanton PG. Hormonal regulation of sertoli cell micro-RNAs at spermiation. Endocrinology. 2011;152(4):1670–83.
135. Panneerdoss S, Chang YF, Buddavarapu KC, Chen HI, Shetty G, Wang H, et al. Androgen-responsive microRNAs in mouse Sertoli cells. PLoS One. 2012;7(7), e41146; PMCID: 3401116.
136. Meunier J, Lemoine F, Soumillon M, Liechti A, Weier M, Guschanski K, et al. Birth and expression evolution of mammalian microRNA genes. Genome Res. 2013;23(1):34–45; PMCID: 3530682.
137. Song R, Ro S, Michaels JD, Park C, McCarrey JR, Yan W. Many X-linked microRNAs escape meiotic sex chromosome inactivation. Nat Genet. 2009;41(4):488–93; PMCID: 2723799.
138. Song R, Hennig GW, Wu Q, Jose C, Zheng H, Yan W. Male germ cells express abundant endogenous siRNAs. Proc Natl Acad Sci U S A. 2011;108(32):13159–64; PMCID: 3156200.
139. Ewen KA, Koopman P. Mouse germ cell development: from specification to sex determination. Mol Cell Endocrinol. 2010;323(1):76–93.
140. Rakoczy J, Fernandez-Valverde SL, Glazov EA, Wainwright EN, Sato T, Takada S, et al. MicroRNAs-140-5p/140-3p modulate Leydig cell numbers in the developing mouse testis. Biol Reprod. 2013;88(6):143.
141. Wainwright EN, Jorgensen JS, Kim Y, Truong V, Bagheri-Fam S, Davidson T, et al. SOX9 regulates microRNA miR-202-5p/3p expression during mouse testis differentiation. Biol Reprod. 2013;89(2):34.
142. Matsui Y. The molecular mechanisms regulating germ cell development and potential. J Androl. 2010;31(1):61–5.
143. de Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl. 2000;21(6):776–98.
144. Huszar JM, Payne CJ. MicroRNA 146 (Mir146) modulates spermatogonial differentiation by retinoic acid in mice. Biol Reprod. 2013;88(1):15.
145. Yang QE, Racicot KE, Kaucher AV, Oatley MJ, Oatley JM. MicroRNAs 221 and 222 regulate the undifferentiated state in mammalian male germ cells. Development. 2013;140(2):280–90; PMCID: 3597206.
146. Tong MH, Mitchell DA, McGowan SD, Evanoff R, Griswold MD. Two miRNA clusters, Mir-17-92 (Mirc1) and Mir-106b-25 (Mirc3), are involved in the regulation of spermatogonial differentiation in mice. Biol Reprod. 2012;86(3):72; PMCID: 3316268.
147. Tong MH, Mitchell D, Evanoff R, Griswold MD. Expression of Mirlet7 family microRNAs in response to retinoic acid-induced spermatogonial differentiation in mice. Biol Reprod. 2011;85(1):189–97; PMCID: 3123386.
148. Mayr F, Heinemann U. Mechanisms of Lin28-mediated miRNA and mRNA regulation—a structural and functional perspective. Int J Mol Sci. 2013;14(8):16532–53; PMCID: 3759924.
149. Zheng K, Wu X, Kaestner KH, Wang PJ. The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol. 2009;9:38; PMCID: 2719617.
150. Gillis AJ, Stoop H, Biermann K, van Gurp RJ, Swartzman E, Cribbes S, et al. Expression and interdependencies of pluripotency factors LIN28, OCT3/4, NANOG and SOX2 in human testicular germ cells and tumours of the testis. Int J Androl. 2011;34(4 Pt 2):e160–74.
151. Aeckerle N, Eildermann K, Drummer C, Ehmcke J, Schweyer S, Lerchl A, et al. The pluripotency factor LIN28 in monkey and human testes: a marker for spermatogonial stem cells? Mol Hum Reprod. 2012;18(10):477–88; PMCID: 3457707.

152. Chakraborty P, Buaas FW, Sharma M, Snyder E, de Rooij DG, Braun RE. LIN28A marks the spermatogonial progenitor population and regulates its cyclic expansion. Stem Cells. 2014;32(4):860–73; PMCID: 4151563.
153. Li M, Yu M, Liu C, Zhu H, He X, Peng S, et al. miR-34c works downstream of p53 leading to dairy goat male germline stem-cell (mGSCs) apoptosis. Cell Prolif. 2013;46(2):223–31.
154. Yu M, Mu H, Niu Z, Chu Z, Zhu H, Hua J. miR-34c enhances mouse spermatogonial stem cells differentiation by targeting Nanos2. J Cell Biochem. 2014;115(2):232–42.
155. Bouhallier F, Allioli N, Lavial F, Chalmel F, Perrard MH, Durand P, et al. Role of miR-34c microRNA in the late steps of spermatogenesis. RNA. 2010;16(4):720–31; PMCID: 2844620.
156. Romero Y, Meikar O, Papaioannou MD, Conne B, Grey C, Weier M, et al. Dicer1 depletion in male germ cells leads to infertility due to cumulative meiotic and spermiogenic defects. PLoS One. 2011;6(10), e25241; PMCID: 3187767.
157. Liang X, Zhou D, Wei C, Luo H, Liu J, Fu R, et al. MicroRNA-34c enhances murine male germ cell apoptosis through targeting ATF1. PLoS One. 2012;7(3), e33861; PMCID: 3316505.
158. Bao J, Li D, Wang L, Wu J, Hu Y, Wang Z, et al. MicroRNA-449 and microRNA-34b/c function redundantly in murine testes by targeting E2F transcription factor-retinoblastoma protein (E2F-pRb) pathway. J Biol Chem. 2012;287(26):21686–98; PMCID: 3381132.
159. Wu J, Bao J, Kim M, Yuan S, Tang C, Zheng H, et al. Two miRNA clusters, miR-34b/c and miR-449, are essential for normal brain development, motile ciliogenesis, and spermatogenesis. Proc Natl Acad Sci U S A. 2014;111(28):E2851–7; PMCID: 4104921.
160. Comazzetto S, Di Giacomo M, Rasmussen KD, Much C, Azzi C, Perlas E, et al. Oligoasthenoteratozoospermia and infertility in mice deficient for miR-34b/c and miR-449 loci. PLoS Genet. 2014;10(10), e1004597; PMCID: 4199480.
161. Yuan S, Tang C, Zhang Y, Wu J, Bao J, Zheng H, et al. mir-34b/c and mir-449a/b/c are required for spermatogenesis, but not for the first cleavage division in mice. Biol Open. 2015;4(2):212–23; PMCID: 4365490.
162. Meikar O, Da Ros M, Kotaja N. Epigenetic regulation of male germ cell differentiation. Subcell Biochem. 2013;61:119–38.
163. Dai L, Tsai-Morris CH, Sato H, Villar J, Kang JH, Zhang J, et al. Testis-specific miRNA-469 up-regulated in gonadotropin-regulated testicular RNA helicase (GRTH/DDX25)-null mice silences transition protein 2 and protamine 2 messages at sites within coding region: implications of its role in germ cell development. J Biol Chem. 2011;286(52):44306–18; PMCID: 3248001.
164. Yu Z, Raabe T, Hecht NB. MicroRNA Mirn122a reduces expression of the posttranscriptionally regulated germ cell transition protein 2 (Tnp2) messenger RNA (mRNA) by mRNA cleavage. Biol Reprod. 2005;73(3):427–33.
165. Bjork JK, Sandqvist A, Elsing AN, Kotaja N, Sistonen L. miR-18, a member of Oncomir-1, targets heat shock transcription factor 2 in spermatogenesis. Development. 2010; 137(19):3177–84.
166. Akerfelt M, Morimoto RI, Sistonen L. Heat shock factors: integrators of cell stress, development and lifespan. Nat Rev Mol Cell Biol. 2010;11(8):545–55; PMCID: 3402356.
167. Wang Y, Medvid R, Melton C, Jaenisch R, Blelloch R. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. Nat Genet. 2007;39(3):380–5; PMCID: 3008549.
168. Huang CC, Yao HH. Inactivation of Dicer1 in Steroidogenic factor 1-positive cells reveals tissue-specific requirement for Dicer1 in adrenal, testis, and ovary. BMC Dev Biol. 2010;10:66; PMCID: 2897782.
169. Kim GJ, Georg I, Scherthan H, Merkenschlager M, Guillou F, Scherer G, et al. Dicer is required for Sertoli cell function and survival. Int J Dev Biol. 2010;54(5):867–75.
170. Hayashi K, Chuva de Sousa Lopes SM, Kaneda M, Tang F, Hajkova P, Lao K, et al. MicroRNA biogenesis is required for mouse primordial germ cell development and spermatogenesis. PLoS One. 2008;3(3), e1738; PMCID: 2254191.

171. Maatouk DM, Loveland KL, McManus MT, Moore K, Harfe BD. Dicer1 is required for differentiation of the mouse male germline. Biol Reprod. 2008;79(4):696–703.
172. Liu D, Li L, Fu H, Li S, Li J. Inactivation of Dicer1 has a severe cumulative impact on the formation of mature germ cells in mouse testes. Biochem Biophys Res Commun. 2012; 422(1):114–20.
173. Korhonen HM, Meikar O, Yadav RP, Papaioannou MD, Romero Y, Da Ros M, et al. Dicer is required for haploid male germ cell differentiation in mice. PLoS One. 2011;6(9), e24821; PMCID: 3174967.
174. Greenlee AR, Shiao MS, Snyder E, Buaas FW, Gu T, Stearns TM, et al. Deregulated sex chromosome gene expression with male germ cell-specific loss of Dicer1. PLoS One. 2012;7(10), e46359; PMCID: 3464243.
175. Wu Q, Song R, Ortogero N, Zheng H, Evanoff R, Small CL, et al. The RNase III enzyme DROSHA is essential for microRNA production and spermatogenesis. J Biol Chem. 2012;287(30):25173–90; PMCID: 3408133.
176. Chang YF, Lee-Chang JS, Imam JS, Buddavarapu KC, Subaran SS, Sinha-Hikim AP, et al. Interaction between microRNAs and actin-associated protein Arpc5 regulates translational suppression during male germ cell differentiation. Proc Natl Acad Sci U S A. 2012;109(15):5750–5; PMCID: 3326518.
177. Johanson TM, Lew AM, Chong MM. MicroRNA-independent roles of the RNase III enzymes Drosha and Dicer. Open Biol. 2013;3(10):130144; PMCID: 3814725.
178. Zimmermann C, Romero Y, Warnefors M, Bilican A, Borel C, Smith LB, et al. Germ cell-specific targeting of DICER or DGCR8 reveals a novel role for endo-siRNAs in the progression of mammalian spermatogenesis and male fertility. PLoS One. 2014;9(9), e107023; PMCID: 4171096.
179. Kaneko H, Dridi S, Tarallo V, Gelfand BD, Fowler BJ, Cho WG, et al. DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. Nature. 2011;471(7338):325–30; PMCID: 3077055.
180. Kanellopoulou C, Muljo SA, Kung AL, Ganesan S, Drapkin R, Jenuwein T, et al. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. Genes Dev. 2005;19(4):489–501; PMCID: 548949.
181. Murchison EP, Partridge JF, Tam OH, Cheloufi S, Hannon GJ. Characterization of Dicer-deficient murine embryonic stem cells. Proc Natl Acad Sci U S A. 2005;102(34):12135–40; PMCID: 1185572.
182. Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, et al. World Health Organization reference values for human semen characteristics. Hum Reprod Update. 2010;16(3):231–45.
183. Sharpe RM. Sperm counts and fertility in men: a rocky road ahead. Science & Society Series on Sex and Science. EMBO Rep. 2012;13(5):398–403; PMCID: 3343360.
184. Sharlip ID, Jarow JP, Belker AM, Lipshultz LI, Sigman M, Thomas AJ, et al. Best practice policies for male infertility. Fertil Steril. 2002;77(5):873–82.
185. Savage T, Peek J, Hofman PL, Cutfield WS. Childhood outcomes of assisted reproductive technology. Hum Reprod. 2011;26(9):2392–400.
186. Batcheller A, Cardozo E, Maguire M, DeCherney AH, Segars JH. Are there subtle genome-wide epigenetic alterations in normal offspring conceived by assisted reproductive technologies? Fertil Steril. 2011;96(6):1306–11; PMCID: 3576017.
187. Chalmel F, Lardenois A, Evrard B, Mathieu R, Feig C, Demougin P, et al. Global human tissue profiling and protein network analysis reveals distinct levels of transcriptional germline-specificity and identifies target genes for male infertility. Hum Reprod. 2012;27(11):3233–48.
188. Liu Y, Niu M, Yao C, Hai Y, Yuan Q, Liu Y, et al. Fractionation of human spermatogenic cells using STA-PUT gravity sedimentation and their miRNA profiling. Sci Rep. 2015;5:8084.
189. Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal fertile men. Lancet. 2002;360(9335):772–7.

190. Jodar M, Kalko S, Castillo J, Ballesca JL, Oliva R. Differential RNAs in the sperm cells of asthenozoospermic patients. Hum Reprod. 2012;27(5):1431–8.
191. Sendler E, Johnson GD, Mao S, Goodrich RJ, Diamond MP, Hauser R, et al. Stability, delivery and functions of human sperm RNAs at fertilization. Nucleic Acids Res. 2013;41(7):4104–17; PMCID: 3627604.
192. Krawetz SA, Kruger A, Lalancette C, Tagett R, Anton E, Draghici S, et al. A survey of small RNAs in human sperm. Hum Reprod. 2011;26(12):3401–12; PMCID: 3212879.
193. Salas-Huetos A, Blanco J, Vidal F, Mercader JM, Garrido N, Anton E. New insights into the expression profile and function of micro-ribonucleic acid in human spermatozoa. Fertil Steril. 2014;102(1):213–22. e4.
194. Ostermeier GC, Miller D, Huntriss JD, Diamond MP, Krawetz SA. Reproductive biology: delivering spermatozoan RNA to the oocyte. Nature. 2004;429(6988):154.
195. Luteijn MJ, Ketting RF. PIWI-interacting RNAs: from generation to transgenerational epigenetics. Nat Rev Genet. 2013;14(8):523–34.
196. Jodar M, Selvaraju S, Sendler E, Diamond MP, Krawetz SA, Reproductive MN. The presence, role and clinical use of spermatozoal RNAs. Hum Reprod Update. 2013;19(6):604–24; PMCID: 3796946.
197. Liu WM, Pang RT, Chiu PC, Wong BP, Lao K, Lee KF, et al. Sperm-borne microRNA-34c is required for the first cleavage division in mouse. Proc Natl Acad Sci U S A. 2012;109(2):490–4; PMCID: 3258645.
198. Platts AE, Dix DJ, Chemes HE, Thompson KE, Goodrich R, Rockett JC, et al. Success and failure in human spermatogenesis as revealed by teratozoospermic RNAs. Hum Mol Genet. 2007;16(7):763–73.
199. Montjean D, De La Grange P, Gentien D, Rapinat A, Belloc S, Cohen-Bacrie P, et al. Sperm transcriptome profiling in oligozoospermia. J Assist Reprod Genet. 2012;29(1):3–10; PMCID: 3252406.
200. Liu T, Cheng W, Gao Y, Wang H, Liu Z. Microarray analysis of microRNA expression patterns in the semen of infertile men with semen abnormalities. Mol Med Rep. 2012;6(3):535–42.
201. Abu-Halima M, Hammadeh M, Schmitt J, Leidinger P, Keller A, Meese E, et al. Altered microRNA expression profiles of human spermatozoa in patients with different spermatogenic impairments. Fertil Steril. 2013;99(5):1249–55. e16.
202. Wang C, Yang C, Chen X, Yao B, Yang C, Zhu C, et al. Altered profile of seminal plasma microRNAs in the molecular diagnosis of male infertility. Clin Chem. 2011;57(12):1722–31.
203. Wu W, Hu Z, Qin Y, Dong J, Dai J, Lu C, et al. Seminal plasma microRNAs: potential biomarkers for spermatogenesis status. Mol Hum Reprod. 2012;18(10):489–97.
204. Belleannee C, Legare C, Calvo E, Thimon V, Sullivan R. microRNA signature is altered in both human epididymis and seminal microvesicles following vasectomy. Hum Reprod. 2013;28(6):1455–67.
205. Wu W, Qin Y, Li Z, Dong J, Dai J, Lu C, et al. Genome-wide microRNA expression profiling in idiopathic non-obstructive azoospermia: significant up-regulation of miR-141, miR-429 and miR-7-1-3p. Hum Reprod. 2013;28(7):1827–36.
206. Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M, et al. Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. J Clin Invest. 2004;114(6):744–54; PMCID: 516273.
207. Morales-Prieto DM, Ospina-Prieto S, Chaiwangyen W, Schoenleben M, Markert UR. Pregnancy-associated miRNA-clusters. J Reprod Immunol. 2013;97(1):51–61.
208. Stern-Ginossar N, Elefant N, Zimmermann A, Wolf DG, Saleh N, Biton M, et al. Host immune system gene targeting by a viral miRNA. Science. 2007;317(5836):376–81; PMCID: 4283197.
209. Guo L, Yang Q, Lu J, Li H, Ge Q, Gu W, et al. A comprehensive survey of miRNA repertoire and 3′ addition events in the placentas of patients with pre-eclampsia from high-throughput sequencing. PLoS One. 2011;6(6), e21072; PMCID: 3120834.

210. Betoni JS, Derr K, Pahl MC, Rogers L, Muller CL, Packard RE, et al. MicroRNA analysis in placentas from patients with preeclampsia: comparison of new and published results. Hypertens Pregnancy. 2013;32(4):321–39.
211. Chu T, Mouillet JF, Hood BL, Conrads TP, Sadovsky Y. The assembly of miRNA-mRNA-protein regulatory networks using high-throughput expression data. Bioinformatics. 2015;11:1780–7.
212. Murri M, Insenser M, Fernández-Durán E, San-Millán JL, Escobar-Morreale HF. Effects of polycystic ovary syndrome (PCOS), sex hormones, and obesity on circulating miRNA-21, miRNA-27b, miRNA-103, and miRNA-155 expression. J Clin Endocrinol Metab. 2013; 98(11):E1835–44.
213. Ding CF, Chen WQ, Zhu YT, Bo YL, Hu HM, Zheng RH. Circulating microRNAs in patients with polycystic ovary syndrome. Hum Fertil. 2015;18(1):22–9.

Chapter 19
microRNAs and Hepatitis B

Yutaka Naito, Yasuhito Tanaka, and Takahiro Ochiya

Abstract The hepatitis B virus (HBV) infection is the leading cause of persistent liver diseases, cirrhosis, and hepatocellular carcinoma (HCC). However, the precise mechanism underlying the development of HBV-related diseases is not fully understood. In addition, the therapeutic strategies for the diseases are less than optimum. microRNAs (miRNAs) are small noncoding RNAs that have been described as a "fine-tuner" in various cellular events. The dysregulation of miRNAs play a role in the development of the cancer as well as viral interference. Recent articles have demonstrated that several miRNAs are deregulated by HBV infection and contribute to viral replication and pathogenesis. Thus, it suggests that the precise mechanism between miRNA and HBV biology will be leading to the development of the novel diagnosis and therapy. This chapter aims to provide the basic principals of miRNAs in development of the HBV-related diseases. We also discuss about the possibility of miRNAs on the clinical application for diagnosis and therapy of HBV-related diseases.

Keywords microRNA • Hepatitis B virus • Hepatocellular carcinoma

Introduction

Basic Knowledge of Hepatitis B Virus Infection

The hepatitis B virus (HBV) is a small enveloped DNA virus belonging to the *Hepadnaviridae* family. It has a strong preference for hepatocyte and causes acute or chronic liver diseases. According to World Health Organization (WHO),

Y. Naito • T. Ochiya (✉)
Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
e-mail: tochiya@ncc.go.jp

Y. Tanaka
Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_19

approximately 2000 million people infected with HBV, more than 240 million people are chronic carriers and about 600,000 patients died each year [1]. In most of HBV infection in adults, it is spontaneously eradicated from their liver. However, about 5 % of adults who aquire HBV and also more than 90 % of neonatants who were transmitted HBV from their mothers become chronic carrier [2]. Several lines of evidences reported that chronic HBV infection causes immune-mediated liver damages [3] and oncogenic changes that result in hepatocellular carcinoma (HCC) [2]. Indeed, more than 50 % of all HCC patients are HBV carriers [4]. There are efficient vaccines and treatment strategies of HBV infection including interferon based therapy and nucleotide analogues [5], however persistent HBV infection is still global health problem. Therefore, better knowledge of the pathological and biological basis of HBV infection is necessary for improved control and treatment.

The HBV genome consists of partially double strand DNA of 3.2 kb including seven open-reading frames [6, 7]. The pre-S/S gene encodes three hepatitis B surface antigens (LHBsAg, MHBsAg, and SHBsAg). The pre-core gene encodes hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg). The P and X coding frame encode the viral specific polymerase and the hepatitis B x protein (HBx), respectively. As the first step of viral life cycle in hepatocyte, the entry of HBV particles into the hepatocyte is likely regulated by glycosaminoglycan and sodium taurocholate cotransporting polypeptide (NTCP) [7–9]. After viral entry, HBV core is delivered into nucleolus and the partially circular DNA genome is converted to a covalently closed circular DNA (cccDNA) molecule.

From a cccDNA, all the viral RNAs are transcribed to maintenance HBV infection. Among these transcripts, the pregenomic RNA (pgRNA) serves as template for reverse transcription and generates the viral DNA. The pgRNA are incorporated into the new assembling nucleocapsids in the cytosol. Reverse transcription of pgRNA are initiated after this process.

The assembled viral core acquires envelope proteins into endoplasmic reticulum and form mature virion that will be secreted from host cell, or back to the nucleus to maintain the cccDNA amplification. If the immune system fails to eradicate the virus, the HBV infection becomes chronic. Eventually, in nucleus, cccDNA is integrated into the host cell chromatin and exist as a minichromosome [7, 10, 11].

microRNAs Expression and Hepatitis B Virus Infection

microRNAs (miRNAs) are small noncoding RNAs of 19–25 nucleotides and play important roles in posttranscriptional repression, deadenylation, and degradation [12]. Through inhibiting target gene translation, miRNAs participate in many cellular processes including development, differentiation, stress response, apoptosis, and proliferation. Recent advances in HBV biology uncovered that several miRNA expressions in host cell are deregulated by HBV infection. For instance, HBx that is a key regulator of HBV infection and viral DNA replication has the ability to deregulate the cellular miRNAs expression. As shown in Table 19.1, we summarized miRNAs that was reported as a dysregulated miRNA by HBV infection.

Table 19.1 Dysregulated cellular miRNAs by HBV infection and their effect on HBV biology, pathogenesis, and carcinogenesis

miRNAs	Target gene	miRNA expressions	Correlation with HBx protein	Ref.
Positive regulator of HBV replication				
miR-1	HDAC4	–		Zhang et al. [14]
miR-15b	HNF1α	Down (in early stage of infection)	Downregulated by HBx	Dai et al. [22]
		Up (in late stage of infection)		
miR-122	Cyclin G1	–	Downregulated by HBx	Wang et al. [28]
	HO-1	–		Qiu et al. [49]
miR-155	C/EBP	Up		Wang et al. [37]
	SOCS1	Up		Su et al. [38]
miR-181a	HLA-A	Up		Liu et al. [39]
miR-372/373	NFIB	Up		Guo et al. [17]
miR-501	HBXIP	Up	Support HBx function	Jin et al. [20]
miR-548ah	INFγR1	Up		Xing et al. [45]
miR-581	Dicer, EDEM1	Down		Wang et al. [50]
Negative regulator of HBV replication				
miR-15a	HBp and HBx	Down	Downregulated by HBx	Wang et al. [51]
miR-17-92 cluster	HBV transcripts	Up		Jung et al. [52]
miR-122	HBV DNA polymerase	Down		Chen et al. [27]
miR-125a-5p	HBsAg	Up		Potenza et al. [29]
		Up	Upregulated by HBx	Mosca et al. [30]
miR-141	PPARα	–		Hu et al. [53]
miR-146a	STAT1	Up		Wang et al. [54]
miR-152	DNMT1	Down	Downregulated by HBx	Huang et al. [32]
miR-199a	HBsAg	Up	Upregulated by HBx	Zhang et al. [55]
miR-205	HBx	Down	Downregulated by HBx	Zhang et al. [7]

(continued)

Table 19.1 (continued)

miRNAs	Target gene	miRNA expressions	Correlation with HBx protein	Ref.
miR-210	HBV pre-S1	Up	Upregulated by HBx	Zhang et al. [55]
miR-1231	HBcAg	Up		Kohno et al. [31]

In this chapter, we will first outline the functional role of miRNAs in the HBV biology and related pathogenesis. Subsequently we will describe the miRNA-based strategies for diagnosis and treatment of the HBV-related diseases.

The Biological Role of microRNA in Life Cycle of Hepatitis B Virus and Effects on the Carcinogenesis

The viruses should take several steps to complete their replication and expansion. Among these period, host cellular defensive mechanisms could be developed, including as cell cycle arrest and viral clearance. Thus, the viruses modulate host cellular environments to promote viral replication and achievement life cycle by taking advantages of the miRNA machinery.

Although HBV is a DNA virus, it has not been verified the HBV encoded miRNAs. Only one viral miRNA were identified by in silico approach [13], but its function was not elucidated. As mentioned above, HBV infection induces the dysregulation of several cellular miRNAs. HBV can utilize such dysregulated miRNAs in host cell to modulate host gene expression and cellular condition to promote viral infection and replication.

miRNAs That Promote HBV Replication

Zhang and their colleagues demonstrate that miR-1 enhances HBV replication and transcription in HCC cell line with integrated HBV genome [14]. They also show that miR-1 represses histone deacetylase 4 (HDAC4) and upregulates FXRα, known as transcriptional factor of HBV gene [15]. Epigenetic modification of HBV cccDNA, such as histone acetylation, involves in HBV gene transcription [16]. Thus, they suggested that miR-1 activated the HBV core promoter transcriptional activity to enhance HBV replication. However, they also showed that miR-1 inhibited cell proliferation activity and induced reverse phenotype of cancer cell.

Because HBV-encoded proteins trigger the changes of host cellular phenotype that confer the hallmarks of cancer [2], the functions of miR-1 in HBV infection seem to be contradicted with hepatocellular carcinogenesis. Guo et al. reported that

miR-372/373 are upregulated in HBV infected liver tissue [17]. They also showed that miR-372/373 directly targeted nuclear factor I/B (NFIB) that plays a crucial role in HBV infection and replication. Importantly, these miRNAs expression was significantly elevated in liver cirrhosis and HBV-related HCC [18, 19]. Although further study must be required, it suggests that these miRNAs might have an important role in pathogenesis of HBV-related diseases.

There are also some miRNAs that suggested to work together with HBV protein for promoting viral replication. Jin et al. reported the miRNA that support to the function of HBx protein in host cell. They demonstrated that miR-501 directly targeted Hepatitis B X-interacting protein (HBXIP) [20]. HBXIP was originally identified for its interaction with HBx protein to repress HBV replication [21]. Thus, they suggested that miR-501 regulates the function of HBx and promote HBV replication through inhibiting HBXIP.

Dai and their colleagues reported that the function of miR-15b in HBV replication and its expression kinetics in mouse model from early infection stage to late infection stage [22]. miR-15b directly inhibited hepatocellular nucleic factor 1 alpha (HNF1α) expression to activate HBV Enhancer I which is enhancer sequence of HBV genes. Consequently, miR-15b can promote HBV replication. However, interestingly, in mouse model of HBV infection, miR-15b expression was sustained at low level by HBx protein in the early stage. On the other hands, in late stage of this mouse model that developed HCC, miR-15 expression was increased. Consistent with these points, it was reported that miR-15b expression was elevated at HCC stage [23, 24]. Although precise mechanisms of miR-15b up-regulation in late stage are not still unclear, it may contribute to persistent infection and pathogenesis of HBV.

miRNAs That Repress HBV Replication

Several miRNAs that negatively regulate HBV replication in host cells are also identified. miR-122 is the well studied miRNA in HBV infection as well as several kinds of liver diseases. miR-122 is highly expressed in normal adult liver and has an essential role in various aspects of the liver function, including hepatocellular growth, differentiation, lipid metabolism, and neoplastic transformation [25]. Interestingly, although miR-122 is essential for HCV infection that is also major etiologic factor of chronic liver diseases and HCC [26], it has a contrary effect on the HBV infection. Chen and colleagues showed that miR-122 might directly target the HBV polymerase expression to repress HBV replication [27]. These opposite function of miR-122 between two viruses may be explained by the fact that miR-122 was negatively regulated by HBx protein. Indeed, Wang et al. demonstrated that miR-122 expression was downregulated in HBV infected patients [28]. Thus, it suggests that miR-122 contribute to viral infection and carcinogenesis and has a potential for clinical application.

On the other hand, Potenza et al. identified miR-125a-5p as a directly regulator of HBV gene expression by using computational approach [29]. In addition, they confirmed that miR-125a-5p directly suppressed the HBsAg expression using HCC cell lines. However, their further study showed that miR-125a-5p expression was enhanced by HBx protein [30]. Zhang and their colleagues identified miR-199a and miR-210 a negative regulator of HBV replication through targeting HBsAg [55]. However, also in this case, these miRNA expressions were increased in HBV transfected cell line.

Kohno et al. reported that miR-1231 upregulated in HBV transfected cell lines and HBV mouse model suppressed HBV replication by targeting HBcAg [31]. These examples are certainly complicated because HBV infection induces the expression of miRNAs inhibiting viral replication. However, these miRNAs behavior on virus replication can be understood by focusing on the evasion of HBV from host defense system. The transition from acute to chronic infection of HBV corresponds to a failure of HBV clearance by host immune systems.

Thus, it is conceivable that these miRNAs may contribute to escape from the HBV eradication of host immune systems by suppressing viral replication. Consequently, they reach a dormant state in the infected hepatocyte and the virus can survive until reactivating the HBV life cycle. Corresponding to this point, several researchers suggest that the cccDNA epigenetic status is closely associated with the evasion of HBV from host immune response. In addition to this point, Huang and colleagues demonstrated that the miR-152 regulates CpG islands methylation status of the cccDNA by targeting the DNA methyltransferase 1 (DNMT1). They also showed the downregulation of miR-152 expression was induced by HBx protein [32].

The Biological Role of microRNA in Host Immune Response and Effect on the Carcinogenesis

To survive and acquire the persistent infection in host organism, the virus should escape from several defence systems of host organism, including the modification of homeostasis, the cell cycle arrest, apoptosis and the destruction of the host cell by immune system. Several reports clarified the important role of miRNAs in the development and function of immune system [33]. miR-155 is an immune-related miRNA and participates in the acute inflammatory response after recognition of pathogens by the toll-like receptors [34, 35]. The upregulation of miR-155 can cause the prolong exposure to inflammation which is causal agent to cancers like HCC [36]. Two recent studies reported the function of miR-155 in carcinogenesis and HBV infection. Using HCC-induced mouse model, Wang and collaborators have demonstrated an oncogenic role of miR-155 at the early stages of the tumorigenesis [37]. While, Su et al. demonstrated that miR-155 suppressed suppressor of cytokine signaling 1 (SOCS1) and promotes the JAK/STAT pathway [38]. They also showed that miR-155 expression downregulates HBx expression [38].

Liu et al. reported the upregulation of several miRNAs including miR-181a in HCC cell line with integrated HBV genome [39]. They also showed that dysregulation of miR-181a might contribute to HBV replication by inhibiting the human leukocyte antigen A (HLA-A). However, it is still unclear whether these miRNAs altered in HBV infected cells can regulate cell-specific regulatory functions and affect directly on the immune cells. Several reports show that the presence of circulating miRNAs and the existence of intercellular nanovesicle-mediated miRNA transfer and its impact on the environmental modulates [40–44]. Therefore, it is conceivable that such secretion machinery of miRNA may have specific regulatory function in immune cells to support viral evasion.

Finally, Xing et al. reported the upregulation of miR-548ah in peripheral blood mononuclear cells (PBMCs) at immune activation phase of chronic Hepatitis B compared with PBMC at immune tolerance phase of chronic Hepatitis B [45]. They also showed that miR-548ah may modulate the function of INF-γ through targeting INF-γ receptor 1 (INF-γR1), thereby promoting viral replication. INF-γ, secreted by active Th1 macrophages and natural killer T cell, is important cytokine to increase immune system activity. Thus, it suggests that miR-548ah may contribute to promote viral infection and evasion from host cell immunity.

microRNA as a Biomarker and Therapeutic Target for HBV Infection

Understanding the precise mechanism of viral infection, antiviral immunity, and carcinogenesis is important to development of the diagnosis and powerful targeted therapeutical strategies. Thus, the functional miRNAs are great advantages as therapy for HBV infection and HBV-related HCC.

There are two miRNAs particularly interested for diagnostic or prognostic applications. Waidmann et al. reported the utility of miR-122 for diagnostic application [43]. They confirmed the difference of nine candidate miRNAs expression level in the different group of HBV infected patient serums by using qRT-PCR methods. As a result, they found that miR-122 expression was higher in HBV infected patients than in healthy donors and could distinguish the different patient groups with or without HBV infection, and inactive carrier patients with high or low HBsAg.

As another miRNA, miR-18a was identified as a potential marker for HBV-related HCC screening [46]. When compared with serum expression level of miR-18a in healthy donor and HBV-related chronic hepatitis or cirrhosis, the significant upregulation of miR-18a expression in HBV-related HCC patient serum was identified. Several reports also suggested using a miRNA panel to improve the specificity of the test [40, 42]. Thus, in addition with the current routinely used markers such as HBsAg, HBeAg, and alanine aminotransferase (ALT), the circulating miRNAs in the patient serum have a significant clinical value for better evaluation of the HBV-infection status, liver injury, and early diagnosis of HCC.

As the therapeutic application of miRNA, the work of Ura's group is valuable for finding therapeutic targets involved in HBV-related HCC [47]. First, they analyzed the difference of miRNA expression between the livers of HBV and HCV-positive patients with HCC. As the results, they could indentify 19 miRNAs that were clearly differentiated between HBV and HCV groups. Among these miRNAs, six miRNAs are specific for HBV and 13 miRNAs are specific for HCV. Moreover, they also performed a pathway analysis of candidate targeted genes using identified miRNAs and revealed that these miRNAs targeted pathways were also able to distinguish the cellular mechanisms altered in HBV or HCV-infected livers [47]. The modification of the cellular pathways related to signal transduction, inflammation and natural killer toxicity, DNA damage, recombination, and cell death was observed. While HCV infection modifies those involved in immune response, antigen presentation, cell cycle, and cell adhesion [47].

Finally, Kota and collaborators reported the therapeutic approach of HCC by using viral delivery of miRNA [48]. By using an adeno-associated virus to deliver miR-26a in a mouse model of HCC, they confirmed whether the administration of miR-26a enables safe and efficient in vivo miRNA gene therapy. This resulted in the successful inhibition of the cancer cell proliferation, induction of the tumor-specific apoptosis, and dramatic protection from disease progression without toxicity.

Conclusion

miRNAs have emerged as novel regulator of cellular gene expression. Several lines of evidences revealed that the specific cellular pathways were dysregulated by HBV infection and might be closely associated with HBV-related pathogenesis. However, despite these great efforts to elucidate the underlying mechanisms of HBV infection and related diseases, the precise mechanisms about them still are largely unknown.

HBV utilizes the specific cellular molecules for their replication and evasion from host immune systems. On the contrary, by a cellular point of view, it is also conceivable that dysregulated pathways induced by HBV infection act as a host defensive system to prevent from invader. HBV is one of the most common infectious causes of liver disease worldwide and is implicated in HCC development.

The present and further investigation will clarify the functional role of miRNA in HBV biology and accumulate the knowledge that will be leading to the development of powerful-targeted therapeutical strategies.

Acknowledgement This study was supported by grants from the Ministry of Health, Labour, and Welfare of Japan.

References

1. Zhao X, Wu Y, Duan J, Ma Y, Shen Z, Wei L, Cui X, Zhang J, Xie Y, Liu J. Quantitative proteomic analysis of exosome protein content changes induced by hepatitis B virus in Huh-7 cells using SILAC labeling and LC-MS/MS. J Proteome Res. 2014;13:5391–402.
2. Arzumanyan A, Reis HM, Feitelson MA. Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma. Nat Rev Cancer. 2013;13:123–35.
3. Protzer U, Maini MK, Knolle PA. Living in the liver: hepatic infections. Nat Rev Immunol. 2012;12:201–13.
4. Parkin DM. The global health burden of infection-associated cancers in the year 2002. Int J Cancer. 2006;118:3030–44.
5. Godon O, Fontaine H, Kahi S, Meritet J, Scott-Algara D, Pol S, Michel M, Bourgine M. Immunological and antiviral responses after therapeutic DNA immunization in chronic hepatitis B patients efficiently treated by analogues. Mol Ther. 2014;22:675–84.
6. Ganem D, Prince AM. Hepatitis B virus infection—natural history and clinical consequences. N Engl J Med. 2004;350:1118–29.
7. Zhang X, Hou J, Lu M. Regulation of hepatitis B virus replication by epigenetic mechanisms and microRNAs. Front Genet. 2013;4:202.
8. Schulze A, Gripon P, Urban S. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. Hepatology. 2007;46:1759–68.
9. Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, Li W. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. eLife. 2012;1, e00049.
10. Bock CT, Schranz P, Schroder CH, Zentgraf H. Hepatitis B virus genome is organized into nucleosomes in the nucleus of the infected cell. Virus Genes. 1994;8:215–29.
11. Newbold JE, Xin H, Tencza M, Sherman G, Dean J, Bowden S, Locarnini S. The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes. J Virol. 1995;69:3350–7.
12. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet. 2010;11:597–610.
13. Jin WB, Wu FL, Kong D, Guo AG. HBV-encoded microRNA candidate and its target. Comput Biol Chem. 2007;31:124–6.
14. Zhang X, Zhang E, Ma Z, Pei R, Jiang M, Schlaak JF, Roggendorf M, Lu M. Modulation of hepatitis B virus replication and hepatocyte differentiation by MicroRNA-1. Hepatology. 2011;53:1476–85.
15. Reese V, Ondracek C, Rushing C, Li L, Oropeza CE, McLachlan A. Multiple nuclear receptors may regulate hepatitis B virus biosynthesis during development. Int J Biochem Cell Biol. 2011;43:230–7.
16. Levrero M, Pollicino T, Petersen J, Belloni L, Raimondo G, Dandri M. Control of cccDNA function in hepatitis B virus infection. J Hepatol. 2009;51:581–92.
17. Guo H, Liu H, Mitchelson K, Rao H, Luo M, Xie L, Sun Y, Zhang L, Lu Y, Liu R, Ren A, Liu S, Zhou S, Zhu J, Zhou Y, Huang A, Wei L, Guo Y, Cheng J. MicroRNAs-372/373 promote the expression of hepatitis B virus through the targeting of nuclear factor I/B. Hepatology. 2011;54:808–19.
18. Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, Shimotohno K. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene. 2006;25:2537–45.
19. Hou J, Lin L, Zhou W, Wang Z, Ding G, Dong Q, Qin L, Wu X, Zheng Y, Yang Y, Tian W, Zhang Q, Wang C, Zhang Q, Zhuang SM, Zheng L, Liang A, Tao W, Cao X. Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma. Cancer Cell. 2011;19:232–43.
20. Jin J, Tang S, Xia L, Du R, Xie H, Song J, Fan R, Bi Q, Chen Z, Yang G, Liu J, Shi Y, Fan D. MicroRNA-501 promotes HBV replication by targeting HBXIP. Biochem Biophys Res Commun. 2013;430:1228–33.

21. Melegari M, Scaglioni PP, Wands JR. Cloning and characterization of a novel hepatitis B virus x binding protein that inhibits viral replication. J Virol. 1998;72:1737–43.
22. Dai X, Zhang W, Zhang H, Sun S, Yu H, Guo Y, Kou Z, Zhao G, Du L, Jiang S, Zhang J, Li J, Zhou Y. Modulation of HBV replication by microRNA-15b through targeting hepatocyte nuclear factor 1alpha. Nucleic Acids Res. 2014;42:6578–90.
23. Chung GE, Yoon JH, Myung SJ, Lee JH, Lee SH, Lee SM, Kim SJ, Hwang SY, Lee HS, Kim CY. High expression of microRNA-15b predicts a low risk of tumor recurrence following curative resection of hepatocellular carcinoma. Oncol Rep. 2010;23:113–9.
24. Liu AM, Yao TJ, Wang W, Wong KF, Lee NP, Fan ST, Poon RT, Gao C, Luk JM. Circulating miR-15b and miR-130b in serum as potential markers for detecting hepatocellular carcinoma: a retrospective cohort study. BMJ Open. 2012;2:e000825.
25. Girard M, Jacquemin E, Munnich A, Lyonnet S, Henrion-Caude A. miR-122, a paradigm for the role of microRNAs in the liver. J Hepatol. 2008;48:648–56.
26. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science. 2005;309:1577–81.
27. Chen Y, Shen A, Rider PJ, Yu Y, Wu K, Mu Y, Hao Q, Liu Y, Gong H, Zhu Y, Liu F, Wu J. A liver-specific microRNA binds to a highly conserved RNA sequence of hepatitis B virus and negatively regulates viral gene expression and replication. FASEB J. 2011;25:4511–21.
28. Wang S, Qiu L, Yan X, Jin W, Wang Y, Chen L, Wu E, Ye X, Gao GF, Wang F, Chen Y, Duan Z, Meng S. Loss of microRNA 122 expression in patients with hepatitis B enhances hepatitis B virus replication through cyclin G(1) -modulated P53 activity. Hepatology. 2012;55:730–41.
29. Potenza N, Papa U, Mosca N, Zerbini F, Nobile V, Russo A. Human microRNA hsa-miR-125a-5p interferes with expression of hepatitis B virus surface antigen. Nucleic Acids Res. 2011;39:5157–63.
30. Mosca N, Castiello F, Coppola N, Trotta MC, Sagnelli C, Pisaturo M, Sagnelli E, Russo A, Potenza N. Functional interplay between hepatitis B virus X protein and human miR-125a in HBV infection. Biochem Biophys Res Commun. 2014;449:141–5.
31. Kohno T, Tsuge M, Murakami E, Hiraga N, Abe H, Miki D, Imamura M, Ochi H, Hayes CN, Chayama K. Human microRNA hsa-miR-1231 suppresses hepatitis B virus replication by targeting core mRNA. J Viral Hepat. 2014;21:e89–97.
32. Huang J, Wang Y, Guo Y, Sun S. Down-regulated microRNA-152 induces aberrant DNA methylation in hepatitis B virus-related hepatocellular carcinoma by targeting DNA methyltransferase 1. Hepatology. 2010;52:60–70.
33. Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD. MicroRNAs: new regulators of immune cell development and function. Nat Immunol. 2008;9:839–45.
34. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A. 2007;104:1604–9.
35. Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, Fabbri M, Alder H, Liu CG, Calin GA, Croce CM. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol. 2007;179:5082–9.
36. Berasain C, Castillo J, Perugorria MJ, Latasa MU, Prieto J, Avila MA. Inflammation and liver cancer: new molecular links. Ann N Y Acad Sci. 2009;1155:206–21.
37. Wang B, Majumder S, Nuovo G, Kutay H, Volinia S, Patel T, Schmittgen TD, Croce C, Ghoshal K, Jacob ST. Role of microRNA-155 at early stages of hepatocarcinogenesis induced by choline-deficient and amino acid-defined diet in C57BL/6 mice. Hepatology. 2009;50:1152–61.
38. Su C, Hou Z, Zhang C, Tian Z, Zhang J. Ectopic expression of microRNA-155 enhances innate antiviral immunity against HBV infection in human hepatoma cells. Virol J. 2011;8:354.
39. Liu Y, Zhao JJ, Wang CM, Li MY, Han P, Wang L, Cheng YQ, Zoulim F, Ma X, Xu DP. Altered expression profiles of microRNAs in a stable hepatitis B virus-expressing cell line. Chin Med J (Engl). 2009;122:10–4.

40. Li LM, Hu ZB, Zhou ZX, Chen X, Liu FY, Zhang JF, Shen HB, Zhang CY, Zen K. Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. Cancer Res. 2010;70:9798–807.
41. Kogure T, Lin WL, Yan IK, Braconi C, Patel T. Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. Hepatology. 2011;54:1237–48.
42. Zhou J, Yu L, Gao X, Hu J, Wang J, Dai Z, Wang JF, Zhang Z, Lu S, Huang X, Wang Z, Qiu S, Wang X, Yang G, Sun H, Tang Z, Wu Y, Zhu H, Fan J. Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. J Clin Oncol. 2011;29:4781–8.
43. Waidmann O, Bihrer V, Pleli T, Farnik H, Berger A, Zeuzem S, Kronenberger B, Piiper A. Serum microRNA-122 levels in different groups of patients with chronic hepatitis B virus infection. J Viral Hepat. 2012;19:e58–65.
44. Arataki K, Hayes CN, Akamatsu S, Akiyama R, Abe H, Tsuge M, Miki D, Ochi H, Hiraga N, Imamura M, Takahashi S, Aikata H, Kawaoka T, Kawakami H, Ohishi W, Chayama K. Circulating microRNA-22 correlates with microRNA-122 and represents viral replication and liver injury in patients with chronic hepatitis B. J Med Virol. 2013;85:789–98.
45. Xing TJ, Xu HT, Yu WQ, Wang B, Zhang J. MiRNA-548ah, a potential molecule associated with transition from immune tolerance to immune activation of chronic hepatitis B. Int J Mol Sci. 2014;15:14411–26.
46. Li L, Guo Z, Wang J, Mao Y, Gao Q. Serum miR-18a: a potential marker for hepatitis B virus-related hepatocellular carcinoma screening. Dig Dis Sci. 2012;57:2910–6.
47. Ura S, Honda M, Yamashita T, Ueda T, Takatori H, Nishino R, Sunakozaka H, Sakai Y, Horimoto K, Kaneko S. Differential microRNA expression between hepatitis B and hepatitis C leading disease progression to hepatocellular carcinoma. Hepatology. 2009;49:1098–112.
48. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, Chang TC, Vivekanandan P, Torbenson M, Clark KR, Mendell JR, Mendell JT. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell. 2009;137:1005–17.
49. Qiu L, Fan H, Jin W, Zhao B, Wang Y, Ju Y, Chen L, Chen Y, Duan Z, Meng S. miR-122-induced down-regulation of HO-1 negatively affects miR-122-mediated suppression of HBV. Biochem Biophys Res Commun. 2010;398:771–7.
50. Wang YQ, Ren YF, Song YJ, Xue YF, Zhang XJ, Cao ST, Deng ZJ, Wu J, Chen L, Li G, Shi KQ, Chen YP, Ren H, Huang AL, Tang KF. MicroRNA-581 promotes hepatitis B virus surface antigen expression by targeting Dicer and EDEM1. Carcinogenesis. 2014;35:2127–33.
51. Wang Y, Jiang L, Ji X, Yang B, Zhang Y, Fu XD. Hepatitis B viral RNA directly mediates down-regulation of the tumor suppressor microRNA miR-15a/miR-16-1 in hepatocytes. J Biol Chem. 2013;288:18484–93.
52. Jung YJ, Kim JW, Park SJ, Min BY, Jang ES, Kim NY, Jeong SH, Shin CM, Lee SH, Park YS, Hwang JH, Kim N, Lee DH. c-Myc-mediated overexpression of miR-17-92 suppresses replication of hepatitis B virus in human hepatoma cells. J Med Virol. 2013;85:969–78.
53. Hu W, Wang X, Ding X, Li Y, Zhang X, Xie P, Yang J, Wang S. MicroRNA-141 represses HBV replication by targeting PPARA. PLoS One. 2012;7:e34165.
54. Wang S, Zhang X, Ju Y, Zhao B, Yan X, Hu J, Shi L, Yang L, Ma Z, Chen L, Liu Y, Duan Z, Chen X, Meng S. MicroRNA-146a feedback suppresses T cell immune function by targeting Stat1 in patients with chronic hepatitisB. J Immunol. 2013;191:293–301.
55. Zhang GL, Li YX, Zheng SQ, Liu M, Li X, Tang H. Suppression of hepatitis B virus replication by microRNA-199a-3p and microRNA-210. Antiviral Res. 2010;88:169–75.

Chapter 20
Circulating microRNAs: The Future of Biomarkers in Anti-doping Field

Nicolas Leuenberger and Martial Saugy

Abstract microRNAs (miRNAs) are small non-coding RNAs that regulate various biological processes. Cell-free miRNAs have been proposed as biomarkers of disease, including diagnosis, prognosis, and monitoring of treatment responses. These circulating miRNAs are highly stable in several body fluids, including plasma and serum; hence, in view of their potential use as novel, non-invasive biomarkers, the profiles of circulating miRNAs have been explored in the field of anti-doping. This chapter describes the enormous potential of circulating miRNAs as a new class of biomarkers for the detection of doping substances, and highlights the advantages of measuring these stable species over other methods that have already been implemented in anti-doping regimes. Incorporating longitudinal measurements of circulating miRNAs into the Athlete Biological Passport is proposed as an efficient strategy for the implementation of these new biomarkers. Furthermore, potential challenges related to the transition of measurements of circulating miRNAs from research settings to practical anti-doping applications are presented.

Keywords Doping • Circulating microRNAs • Biomarkers • Athlete biological passport

Introduction

One of the major challenges in the anti-doping field is the identification of specific and sensitive non-invasive biomarkers that can be routinely measured in easily accessible samples. microRNAs (miRNAs) are a particularly promising class of biomarkers; these small (22 nucleotide), non-protein-encoding RNAs post-transcriptionally regulate gene expression via suppression of specific target mRNA [1]. Since their discovery in the early 1990s, miRNAs have been shown to play

N. Leuenberger (✉) • M. Saugy
Swiss Laboratory for Doping Analyses, University Center of Legal Medicine, Lausanne-Geneva, CHUV, University of Lausanne, Switzerland

Lausanne University Hospital, University of Lausanne, Epalinges, Switzerland
e-mail: Nicolas.Leuenberger@chuv.ch

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_20

important regulatory roles in a wide range of biological and pathological processes. Over 500 miRNAs have been identified in humans to date [2]; this number seems limited compared with the estimated 30,000 conventional messenger RNAs in humans [3].

In 2008, miRNAs were identified in serum, plasma, urine, saliva, and other body fluids. Although RNA molecules are generally unstable, circulating miRNAs are highly stable and readily detectable. The mechanism underlying the remarkable stability of circulating miRNAs in blood has been investigated and discussed intensively [4]. Emerging evidence has demonstrated that miRNAs in the serum remain stable after being subjected to severe conditions that would degrade most other miRNAs. Despite the presence of RNase activity in plasma, subjecting plasma samples to up to eight freeze-thaw cycles or incubating them at room temperature for up to 24 h has only a minimal effect on circulating miRNA levels [4]. By contrast, synthetic miRNAs added directly to plasma are degraded rapidly, suggesting that endogenous circulating miRNAs exist in a form that is resistant to plasma RNase activity [4]. Differential centrifugation and size-exclusion chromatography experiments identified two populations of miRNAs in blood; most circulating miRNAs co-fractionate with complexes of the argonaute 2 protein, whereas a specific minority are predominantly associated with vesicles [5, 6]. Both types of circulating miRNAs are sensitive to protease treatment of plasma, but are protected against digestion by plasma RNase [5]. The identification of extracellular argonaute 2-miRNA complexes in plasma raises the possibility that cells release a functional miRNA-induced silencing complex into the blood.

Circulating miRNAs in the Anti-doping Field

In the same way that certain biomarkers are used to detect the biological fingerprint of disease, other biomarkers can be used to detect the biological fingerprint of doping. The potential use of circulating miRNAs as specific biomarkers in the anti-doping field has been demonstrated by several groups. Leuenberger et al. found that a single injection of continuous erythropoietin receptor activator induces an increase in the plasma level of miR-144, a miRNA that plays an essential role in erythropoiesis in a number of organisms [7]. This finding emphasised the potential use of miRNAs as circulating biomarkers for the detection of erythropoiesis-stimulating agents. In addition, Kelly and colleagues found that four miRNAs are differentially expressed in individuals using therapeutic replacement doses of recombinant human growth hormone compared with normal controls or individuals with naturally high levels of growth hormone, suggesting that circulating miRNAs may be used as biomarkers for the detection of recombinant human growth hormone abuse [8].

In a recent study, we demonstrated that circulating miRNAs can also be used to detect autologous blood transfusion [9]. In this study, transfusion of autologous blood triggered increases in the levels of several miRNAs in plasma. Because the blood transfusion caused large increases in the levels of miRNAs in the lungs, the

origin of these circulating miRNAs was likely pulmonary tissues. In addition, a partial suppression of erythropoietin was observed after the transfusion; hence, a mathematical model that used a combination of miRNA and erythropoietin levels enhanced the efficiency of autologous transfusion detection.

The use of circulating miRNAs to detect performance-enhancing agents could also be extrapolated to other doping trends, such as testosterone abuse. Currently, the gold standard method of detecting exogenous testosterone administration is gas chromatography–mass spectrometry analyses of urine samples. However, measurements of circulating miRNAs in plasma and serum are used to detect testosterone abuse in our laboratory, and preliminary results show that longitudinal measurements of miRNA levels in plasma could be a promising approach.

Methods for the Detection of Circulating miRNAs

Serum, plasma, and whole blood have been assessed as matrices for miRNA profiling in blood. The choice of matrix has a significant effect on miRNA concentrations; for example, serum has lower amounts of circulating miRNAs than plasma [10]. Because of the high number of blood analyses required for the Athlete Biological Passport (ABP), EDTA-plasma is the most widely used matrix in the anti-doping field [11]. Because blood parameters and miRNAs can be measured in the same matrix, a single plasma-EDTA sample would be sufficient to perform the tests. Thus plasma seems to be the material of choice for miRNA analyses in anti-doping laboratories. However, plasma samples containing heparin as an anticoagulant should not be used because this substance can interfere with downstream assays, such as reverse transcription (RT) of the RNA prior to PCR analyses.

A critical factor that might affect the results of miRNA quantification is the presence of haemolysis. Several studies demonstrate that mature erythrocytes contain abundant and diverse miRNA species. In a recent report, Blondal et al. described a number of different protocols and tests to assess haemolysis [12]. A simple and cost-effective method is to measure oxyhaemoglobin absorbance at $\lambda = 414$ nm using a spectrophotometer. Other methods based on a comparison of the levels of miR-451, which is expressed at a high level in erythrocytes, and miR-23a, which is not affected by haemolysis, have also been proposed [12].

Because of the small amount of circulating miRNAs and the large amounts of proteins and lipids, extraction of miRNAs from blood samples is technically challenging. Moreover, plasma and serum contain various RT and PCR inhibitors. To overcome these challenges, several phenol/chloroform-based protocols for the extraction of miRNAs from biological fluids have been developed [13]. Most of these methods combine phenol/guanidine-based lysis of samples and silica membrane-based purification of total RNA. Typically, a monophasic solution of phenol and guanidine thiocyanate is used to facilitate lysis, denature protein complexes and RNases, and remove most of the residual DNA and proteins from the lysate by organic extraction. After the addition of chloroform, the lysate is separated

into the aqueous and organic phases by centrifugation. The aqueous phase is then extracted and the sample is applied to a silica membrane-based spin column, to which the total RNA binds but phenol and other contaminants are efficiently washed away. In addition, Andreasen et al. demonstrated that the addition of a small carrier RNA prior to total RNA extraction improves the yield and reproducibility of phenol/chloroform-based RNA extractions [13].

Commercially available kits that do not use acid phase separation can also be used to isolate miRNAs from body fluids. The general principle of extraction of these kits is similar to that of the phenol/chloroform method, but the non-phenol lysis solution contains guanidine thiocyanate and a salt solution to denature and precipitate proteins, respectively. The phenol/chloroform procedure typically produces higher yields of miRNAs than non-phenol-based lysis procedures, displays reduced variance between assays, and can be fully automated; therefore, it is likely the optimal method for the extraction of miRNAs from biofluids [10]. Nevertheless, different extraction methods should be tested to find the most suitable procedure for each study.

One of the main problems associated with circulating miRNA extraction and sample comparisons is the accurate quantification of the miRNAs. The low abundance of miRNAs in plasma is barely detectable using spectrophotometry. The efficiency of extraction of circulating miRNAs can be assessed by spiking the sample with known quantities of non-human miRNAs (such as those from *Caenorhabditis elegans*). The synthetic spike-in miRNAs are added to the denaturation buffer (TRIzol or lysis buffer) and subjected to the entire extraction process. The amount of spike-in miRNA in the eluate is typically measured by quantitative RT-PCR, thereby providing an internal reference to assess the efficiency of the extraction. Furthermore, the addition of synthetic spike-in miRNA can also be used to assess the efficiency of the RT reaction.

Quantitative RT-PCR is the gold standard method of quantifying circulating miRNAs. This technique has high sensitivity and specificity and is suitable for quantification across a wide dynamic range (the ratio between the largest and smallest values of a changeable quantity). Two major strategies enable the specific amplification of mature miRNAs via RT-PCR. In the first strategy, a stem-loop-shaped primer binds specifically to the mature miRNA to generate a unique template for RT [14]. Alternatively, in poly(A)-tailed PCR, a poly(A) tail is added to the 3′ UTR of a mature miRNA by a polyadenylating enzyme, and then universal primers containing 5′ terminal oligo (dT) sequences are used to initiate the RT reaction [15].

Although the technical variability of circulating miRNA extraction can be normalised by using a non-human spiked-in control, no absolute internal controls exist due to the cell-free condition of the sample. Invariant (non-changing) miRNAs are sometimes selected as endogenous controls; however, the biological significance of specific miRNAs can be underestimated using this approach [15]. For example, miR-16 is frequently used as an endogenous control because it is expressed at a high level in plasma and is relatively invariant across a large number of samples [15]. However, elevated levels of miR-16 in serum correlate with several pathologies; hence, further investigation is required to ensure adequate normalisation in this

scenario [16]. The low-throughput nature of quantitative PCR is also a key issue for miRNA-based studies; however, recently developed PCR array platforms have partly solved this problem. For example, Exiqon produce miRNA Ready-to-Use PCR arrays, which are 384-well microfluidics cards containing preloaded miRNA-specific PCR primers; hence these arrays can profile hundreds of miRNAs simultaneously. Several other medium-throughput platforms, including customisable plates, are also commercially available [3].

Potential Use of Circulating miRNAs as a Component of the ABP

The fight against doping is based mainly on the direct detection of a prohibited substance in an athlete's biological sample. In addition, doping triggers physiological changes that provide enhancements in performance; therefore, some methods, such as the ABP, use indirect markers of doping [11]. In 2008, the haematological module of the ABP was the first to be implemented by the International Cycling Union [17]. To identify patterns related to blood doping, such as exposure to erythropoiesis-stimulating agents or autologous blood transfusion, biomarkers related to the haematopoietic system, including the haemoglobin concentration and reticulocyte levels, are monitored over time and analysed using mathematical models that quantify individual variation [18].

The ABP, a new paradigm with immense potential in the current climate of rapid advancements in biomarker discovery [11], uses information from biological tests as indirect evidence to detect doping. Blood is a living tissue that undergoes permanent changes over time, and these changes must be taken into account during evaluation of the data or limited by implementing standardised procedures. For this reason, stringent processes for sample collection, transport, and analysis of the data have been put into place to guarantee the objective and reliable use of the ABP [19].

The use of circulating miRNAs to detect blood doping could be incorporated into the adaptive model of the ABP [20]. Among the advantages of miRNAs as biomarkers is their high stability in blood. In addition, in contrast to other haematological parameters, the levels of circulating miRNAs are not affected by environmental factors such as inadequate storage during transport of the blood samples [21]; therefore, the requirement for cumbersome documentation regarding the transport and storage of samples could be avoided. In addition, cDNA samples are highly stable in different storage conditions [22].

Blood samples included in the haematological modules of the ABP are analysed in laboratories that are accredited by the World Anti-Doping Agency, and strict quality control criteria are applied to ensure that analyses are executed at a forensic quality level. Blood parameter analyses are performed using automated blood cell counters, and the resulting haematologic variables could be correlated with quantitative PCR measurements of circulating miRNA levels. PCR-based detection of miRNAs is a well-established, robust, and reproducible method that has a number

of key advantages, including its high sensitivity and specificity, potential for target multiplexing, and low RNA input requirement, all of which facilitate expression analyses, even in anti-doping samples with limited amounts of material [12]. In addition, this technology has already been implemented in a large number of diagnostic laboratories and routine protocols are well established. Quality controls could easily be included at every step to monitor sample haemolysis as well as extraction, RT, and qPCR efficiencies, and could be used as normalisation factors [12]. The incorporation of quality controls eliminates the within-subject variation and increases the efficiency and accuracy of evaluation of biological data.

Rigorous procedures are required to ensure the proper implementation and validation of new biomarkers in the ABP. For the haematological module, it was possible to take advantage of extensive prior experience with biomarkers such as the haematocrit and haemoglobin level. For example, Schumacher et al. demonstrated that the haemoglobin concentration shows significant diurnal- and exercise-related variations [23]. The same group also reported that the percentage of reticulocytes in blood samples is modified by long- and short-term exercise [24]. These observations are taken into account during the interpretation of blood profiles of athletes. However, little data currently exists regarding external or environmental factors that could interfere with miRNA measurements in blood. Nevertheless, different laboratories have demonstrated that exercise can influence certain types of circulating miRNAs, such as miRNAs involved in inflammation and muscle metabolism [25–31]. In addition, the impacts of other confounding factors, such as altitude and the use of hypoxic chambers, on circulating miRNAs levels should also be studied. In contrast to haemoglobin, circulating miRNAs were only discovered fairly recently (in 2008); therefore, the results of additional studies in different research areas, such as the diagnosis of diseases based on measurements of circulating miRNAs as blood-related biomarkers, should be incorporated into the interpretation of longitudinal miRNA measurements in the context of the ABP.

Conclusions and Future Perspectives

The enormous potential of circulating miRNAs as a class of anti-doping biomarkers is based on a number of advantageous features of these species. First, circulating miRNAs are remarkably stable molecules, are well preserved in harsh conditions, and are resistant to RNase activity. In addition, they are easily accessible, can be sampled in a relatively non-invasive manner, and are readily measured by simple quantitative PCR approaches. The incorporation of longitudinal measurements of circulating miRNAs into the ABP should enable their implementation as new biomarkers in the anti-doping field. Nonetheless, the field of circulating miRNA research is still in its infancy and further work must be done to characterise potential confounding factors that may influence their use as reliable biomarkers. Because of the plethora of data supporting the clinical use of miRNAs as biomarkers of diseases, a number of robust and reproducible tests for use in patient treatment

decisions are currently under development. Thus clinical information related to the use of circulating miRNAs as a new generation of biomarkers could be extrapolated to anti-doping research.

References

1. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem. 2010;79:351–79; Epub 2010/06/11. eng.
2. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet. 2010;11(9):597–610; Epub 2010/07/28. eng.
3. Pritchard CC, Cheng HH, Tewari M. MicroRNA profiling: approaches and considerations. Nat Rev Genet. 2012;13(5):358–69; Epub 2012/04/19. eng.
4. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105(30):10513–8; PMID: MID 18663219. eng.
5. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A. 2011;108(12):5003–8; PMID: MID 21383194. eng.
6. Chevillet JR, Kang Q, Ruf IK, Briggs HA, Vojtech LN, Hughes SM, et al. Quantitative and stoichiometric analysis of the microRNA content of exosomes. Proc Natl Acad Sci U S A. 2014;111(41):14888–93; PMCID: 4205618.
7. Leuenberger N, Jan N, Pradervand S, Robinson N, Saugy M. Circulating microRNAs as long-term biomarkers for the detection of erythropoiesis-stimulating agent abuse. Drug Test Anal. 2011;3(11-12):771–6; Epub 2011/11/25. eng.
8. Kelly BN, Haverstick DM, Lee JK, Thorner MO, Vance ML, Xin W, et al. Circulating microRNA as a biomarker of human growth hormone administration to patients. Drug Test Anal. 2014;6(3):234–8. doi:10.1002/dta.1469; Epub 2013/03/16. Eng.
9. Leuenberger N, Schumacher YO, Pradervand S, Sander T, Saugy M, Pottgiesser T. Circulating microRNAs as biomarkers for detection of autologous blood transfusion. PLoS One. 2013;8(6), e66309.
10. McDonald JS, Milosevic D, Reddi HV, Grebe SK, Algeciras-Schimnich A. Analysis of circulating microRNA: preanalytical and analytical challenges. Clin Chem. 2011;57(6):833–40; PMID: MID 21487102. Eng.
11. Sottas PE, Vernec A. Current implementation and future of the Athlete Biological Passport. Bioanalysis. 2012;4(13):1645–52; Epub 2012/07/27. eng.
12. Blondal T, Jensby Nielsen S, Baker A, Andreasen D, Mouritzen P, Wrang Teilum M, et al. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. Methods. 2013;59(1):S1–6; Epub 2012/10/06. eng.
13. Andreasen D, Fog JU, Biggs W, Salomon J, Dahslveen IK, Baker A, et al. Improved microRNA quantification in total RNA from clinical samples. Methods. 2010;50(4):S6–9; Epub 2010/03/11. eng.
14. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005;33(20), e179; PMCID: 1292995.
15. Kroh EM, Parkin RK, Mitchell PS, Tewari M. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). Methods. 2010;50(4):298–301; Epub 2010/02/12. eng.
16. Schwarzenbach H, Nishida N, Calin GA, Pantel K. Clinical relevance of circulating cell-free microRNAs in cancer. Nat Rev Clin Oncol. 2014;11(3):145–56.

17. Schumacher YO, Saugy M, Pottgiesser T, Robinson N. Detection of EPO doping and blood doping: the haematological module of the Athlete Biological Passport. Drug Test Anal. 2012;4(11):846–53; Epub 2012/03/01. eng.
18. Pottgiesser T, Sottas PE, Echteler T, Robinson N, Umhau M, Schumacher YO. Detection of autologous blood doping with adaptively evaluated biomarkers of doping: a longitudinal blinded study. Transfusion. 2011;51(8):1707–15; Epub 2011/03/09. eng.
19. Robinson N, Sottas PE, Pottgiesser T, Schumacher YO, Saugy M. Stability and robustness of blood variables in an antidoping context. Int J Lab Hematol. 2011;33(2):146–53; Epub 2010/08/20. eng.
20. Leuenberger N, Robinson N, Saugy M. Circulating miRNAs: a new generation of anti-doping biomarkers. Anal Bioanal Chem. 2013;405(30):9617–23.
21. Grasedieck S, Sorrentino A, Langer C, Buske C, Dohner H, Mertens D, et al. Circulating microRNAs in hematological diseases: principles, challenges and perspectives. Blood. 2013;121:4977–84; Epub 2013/04/04. Eng.
22. Mraz M, Malinova K, Mayer J, Pospisilova S. MicroRNA isolation and stability in stored RNA samples. Biochem Biophys Res Commun. 2009;390(1):1–4; Epub 2009/09/23. eng.
23. Schumacher YO, Wenning M, Robinson N, Sottas PE, Ruecker G, Pottgiesser T. Diurnal and exercise-related variability of haemoglobin and reticulocytes in athletes. Int J Sports Med. 2010;31(4):225–30; Epub 2010/02/12. eng.
24. Schumacher YO, Sahm D, Baumstark MW, Pottgiesser T. Reticulocytes in athletes: Longitudinal aspects and the influence of long- and short-term exercise. Drug Test Anal. 2010;2(10):469–74; Epub 2010/10/05. eng.
25. Baggish AL, Hale A, Weiner RB, Lewis GD, Systrom D, Wang F, et al. Dynamic regulation of circulating microRNA during acute exhaustive exercise and sustained aerobic exercise training. J Physiol. 2011;589(Pt 16):3983–94; PMCID: 3179997; Epub 2011/06/22. eng.
26. Nielsen S, Akerstrom T, Rinnov A, Yfanti C, Scheele C, Pedersen BK, et al. The miRNA plasma signature in response to acute aerobic exercise and endurance training. PLoS One. 2014;9(2), e87308; PMCID: 3929352.
27. Wu XD, Zeng K, Liu WL, Gao YG, Gong CS, Zhang CX, et al. Effect of aerobic exercise on miRNA-TLR4 signaling in atherosclerosis. Int J Sports Med. 2014;35(4):344–50.
28. Pareja-Galeano H, Sanchis-Gomar F, Garcia-Gimenez JL. Physical exercise and epigenetic modulation: elucidating intricate mechanisms. Sports Med. 2014;44(4):429–36.
29. Uhlemann M, Mobius-Winkler S, Fikenzer S, Adam J, Redlich M, Mohlenkamp S, et al. Circulating microRNA-126 increases after different forms of endurance exercise in healthy adults. Eur J Prev Cardiol. 2014;21(4):484–91.
30. Banzet S, Chennaoui M, Girard O, Racinais S, Drogou C, Chalabi H, et al. Changes in circulating microRNAs levels with exercise modality. J Appl Physiol. 2013;115(9):1237–44.
31. Kirby TJ, McCarthy JJ. MicroRNAs in skeletal muscle biology and exercise adaptation. Free Radic Biol Med. 2013;64:95–105.

Chapter 21
microRNA Expression Profiling: Technologies, Insights, and Prospects

Christine Roden*, Stephen Mastriano*, Nayi Wang*, and Jun Lu

Abstract Since the early days of microRNA (miRNA) research, miRNA expression profiling technologies have provided important tools toward both better understanding of the biological functions of miRNAs and using miRNA expression as potential diagnostics. Multiple technologies, such as microarrays, next-generation sequencing, bead-based detection system, single-molecule measurements, and quantitative RT-PCR, have enabled accurate quantification of miRNAs and the subsequent derivation of key insights into diverse biological processes. As a class of ~22 nt long small noncoding RNAs, miRNAs present unique challenges in expression profiling that require careful experimental design and data analyses. We will particularly discuss how normalization and the presence of miRNA isoforms can impact data interpretation. We will present one example in which the consideration in data normalization has provided insights that helped to establish the global miRNA expression as a tumor suppressor. Finally, we discuss two future prospects of using miRNA profiling technologies to understand single cell variability and derive new rules for the functions of miRNA isoforms.

These authors are contributed equally.

C. Roden • S. Mastriano
Department of Genetics, Yale Stem Cell Center and Yale Cancer Center, Yale University School of Medicine, 10 Amistad Street, Rm 237C, New Haven, CT 06520-8005, USA

N. Wang
The Biomedical Engineering Graduate Program, New Haven, CT 06520, USA

J. Lu (✉)
Department of Genetics, Yale Stem Cell Center and Yale Cancer Center, Yale University School of Medicine, 10 Amistad Street, Rm 237C, New Haven, CT 06520-8005, USA

Yale Center for RNA Science and Medicine, New Haven, CT 06520, USA
e-mail: jun.lu@yale.edu

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_21

miRNA Profiling Technologies and Considerations on miRNA Isoforms

After the initial discoveries of *lin-4* and *let-7* miRNAs in *C. elegans* [1–3], searches for similar functional small RNAs have led to the realization that hundreds to thousands of miRNAs exist in genomes ranging from worms to human, which are now well annotated in the online database miRBase [4]. To quantify the expression of many mature miRNAs simultaneously and economically, multiple miRNA expression profiling technologies have been developed. Unlike measuring longer RNAs, measurements of miRNA expression faces a unique challenge of how to detect ~22 nucleotide small RNAs specifically and accurately, being that the short length of mature miRNAs makes it difficult to use regular reverse transcription procedures.

Two major approaches have been utilized to address the difficulty of detecting mature miRNAs. One approach extends the length of miRNA molecules to permit easier reverse transcription, either through adaptor ligation or 3′ tailing of additional nucleotides. Adaptor ligation is one of the most common methods to prepare small RNA libraries for microarray, bead-based detection, or next-generation sequencing analyses [5–7]. The most common adaptor ligation protocols require ligating both an adaptor on the 3′ end of the miRNA, catalyzed by an RNA ligase, and another adaptor on the 5′ end of the miRNA (Fig. 21.1). The presence of the ligated adaptors on the miRNAs provides molecular handles for successful priming of reverse transcription reactions and PCR. Another strategy is to extend the 3′ end of miRNA molecules by tailing with additional nucleotides, often catalyzed by a terminal nucleotidyl transferase. For example, an artificially added poly A tail allows the binding of a poly-dT primer for reverse transcription. The second major approach utilizes various methods of direct hybridization. Because mature miRNAs are of short lengths, conventional hybridization based miRNA-detection protocols face the challenge of lower hybridization temperature and hence potential lower specificity. Two examples to solve this challenge involve either chemically modified oligonucleotides with higher annealing temperatures [8] or hybridization of two short oligonucleotides to the same molecule of miRNA, thus creating a stacking effect that improves the specificity [9, 10] The pros and cons of each specific technology have been examined and discussed carefully elsewhere [11] and will not be a major topic in this chapter.

A key issue that users of these technologies face is the analysis and interpretation of small RNA profiling data. Many researchers new to the miRNA field mistakenly take the face values of the profiling results as a direct reflection of mature miRNA expression levels within cells. It is thus important to emphasize that the existence of mature miRNA isoforms can complicate the interpretation of profiling results. MiRNA isoforms can be classified into two categories: (1) sequence isoforms and (2) biochemical-modification isoforms. Deep sequencing data on small RNAs (e.g., [7, 12]) and northern blot analyses of specific miRNAs (e.g., [7, 12, 13]) have revealed that multiple sequence isoforms of the same miRNA coexist in the cells. These isoforms may differ in length, but may also differ in the start position of the

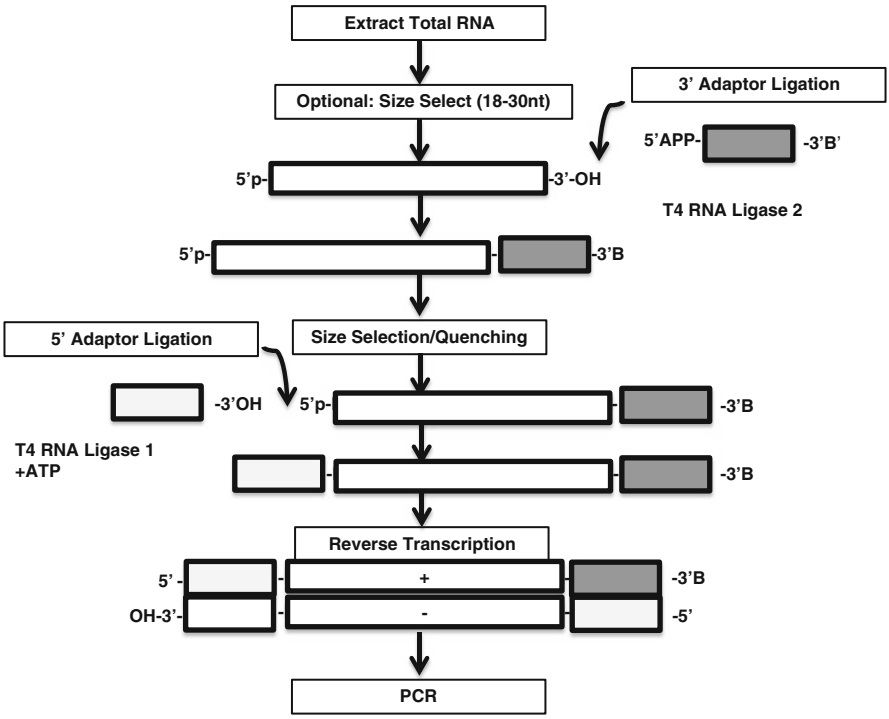

Fig. 21.1 Adaptor-ligation-based method for miRNA amplification and quantification. T4 RNA ligase catalyzed addition of single-stranded 5′ and 3′ adaptors is a frequently used approach to amplify miRNAs and quantify their expression. Among these steps, the 3′ adaptor is blocked on the 3′ end to avoid self-ligation. 3′ adaptor is often pre-adenylated on the 5′ end (5′App) which allows reaction with miRNAs in the absence of ATP, to avoid circularization of miRNAs

5′ nucleotide and/or the end position of the 3′ nucleotide. For example, miR-222 isoform expression in the Hodgkin's lymphoma cell line HDMYZ is shown in Fig. 21.2. Variability during miRNA biogenesis is one of the major sources of variations in mature miRNA sequence length and start/end positions. miRNAs are transcribed as relatively long hairpin-bearing primary miRNA (pri-miRNA). Such pri-miRNA molecules are processed, often co-transcriptionally, by Drosha/DGCR8-containing microprocessor complex within nucleus [14, 15] into precursor miRNA (pre-miRNA). After transport out of the nucleus, pre-miRNAs are then processed by the Dicer/TRBP complex into mature miRNAs. Both DGCR8/Drosha and TRBP/Dicer processing steps are known to generate some level of variability in the processing position on miRNA hairpin [16, 17]. Another source of mature miRNA variation is post-processing modification of mature miRNAs. For example, when miRNAs bind to their targets with sufficient complementarity, both target-template-based tailing and trimming may occur [18], resulting in multiple miRNA isoforms with differing 3′ ends. In addition, non-templated nucleotide additions at 3′ end of miRNAs have also been well documented [19–22].

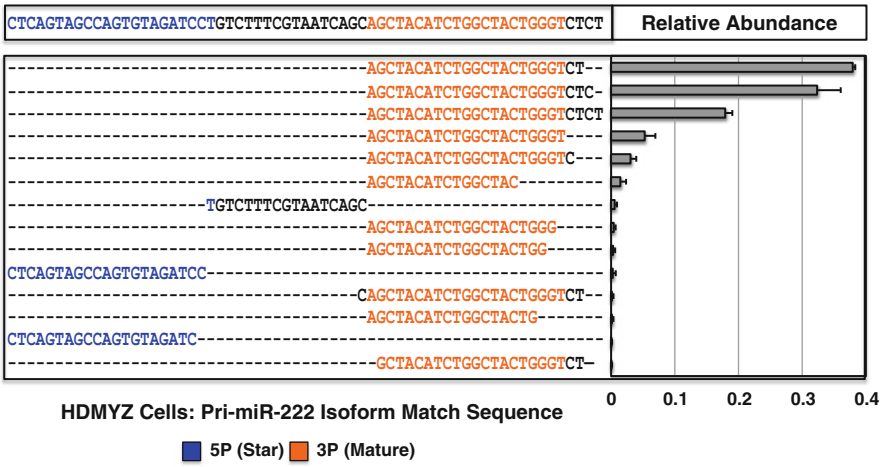

Fig. 21.2 Example of miRNA sequence variations. Small RNA sequencing data in HDMYZ cells were analyzed to demonstrate sequence variability of miR-222-5p and miR-222-3p. Sequences for miRNA precursor are shown on the *top*, with mature miRNAs in miRBase (version 21) in *colored fonts*. The isoforms and their relative abundance are shown, with the total miR-222 level set to one. *Error bars* represent standard deviation ($n = 3$)

Given that various miRNA expression profiling technology utilize different biochemical principles for miRNA labeling and/or detection, any given technology can only detect a specific subset of miRNA isoforms, and may produce inaccurate measurements for some miRNAs in cell. For example, several miRNA profiling technologies, such as the detection methodology utilized in a specific microarray platform [23, 24], require precise 3′ ending position of the miRNAs to match those of the designed probe sequences. The most common practice is to design such miRNA detection platforms with miRNA sequences from miRBase. Although in the majority of cases the start and end positions of a given miRBase miRNA reflect what are expressed in cells, there are plenty of instances in which the miRBase mature miRNA sequence differ from the most abundant miRNA isoforms in a cell type of interest. For example, in HDMYZ cells, the most abundant isoform of miR-222-3p differs 2 bases from the corresponding miRBase (Release 21) sequence at the 3′ end (Fig. 21.2) [25]. It is thus conceivable that for some miRNAs, those technologies requiring precise 3′ end of miRNAs may end up detecting a minor isoform not functionally important in cells. It is also important to note that validation techniques, such as quantitative RT-PCR, can have similar requirements for the 3′ end positions of miRNAs, which, again, are usually designed based on miRBase sequences. For example, the reverse transcription step of TaqMan qRT-PCR [26] will not allow the detection of miRNA molecules with a 3′ end extending beyond those of their designed sequences [20, 21].

Other than isoforms of different lengths and positions, miRNA isoforms with the same sequence but different biochemical features also coexist in the same cells.

As a product of RNase III enzymes, newly produced mature miRNAs should bear a 5′ phosphate group and have a 3′-hydroxyl group. The 5′ phosphate group is important for the positioning of the mature miRNA within Argonaute (AGO) proteins [27, 28], which is an essential component of the RNA-mediated silencing complex. However, dephosphorylation can occur in the presence of phosphatases [29], leading to a mixture of both phosphorylated and non-phosphorylated miRNA molecules in the same cells. In addition to 5′ biochemical isoforms that differ at the level of phosphorylation, 3′ chemical modifications have also been reported to occur on miRNAs [20].

A large number of miRNA detection technologies, including most qRT-PCR methods and many profiling techniques, do not require the presence of 5′-phosphate for detection, and thus will report the total abundance of miRNA isoforms, regardless of their phosphorylation status. Given that the presence of 5′ phosphate on miRNA molecules determine its interaction with AGO proteins [28] and thus may impact its interaction with their targets, reporting total miRNA isoform abundance without considering miRNA phosphorylation status can lead to key missing information for follow-up studies. Among miRNA profiling methods, most adaptor ligation-based library preparation techniques (Fig. 21.1) do require the presence of 5′ phosphate groups for successful 5′ ligation reactions, and can provide expression information for miRNAs with 5′ phosphorylation.

Considerations on the Normalization of miRNA Expression Profiling Data

Data normalization is a critical step that can significantly impact the proper interpretation of miRNA expression profiling data. Almost every method of data normalization is associated with hidden assumptions that are not always explicitly detailed. For genome-wide protein-coding transcriptome analysis, there are a few frequently used normalization methods. These include the assumption of similar distribution of the expression levels in the transcriptome across samples (such as the RMA method), the assumption of total or median expression of transcripts being equal across samples (such as using RPKM measurements for RNA-seq analyses), and the assumption that the expression levels of invariant gene expression are similar across samples (such as using “house-keeping” gene expression for qRT-PCR normalization).

However, these assumptions used for protein-coding transcripts may not be applicable to miRNA studies. In most biological samples, the number of detectable miRNAs is usually in the low hundreds, with the majority of miRNAs documented in miRBase having no detectable expression. Thus, cautions should be applied with median-based normalization to ensure that experimental noise will not heavily influence final normalization results. Similarly, it cannot always be assumed that the distribution of miRNA expression is similar across samples, and quantile-based normalization methods should be used with careful validation. In addition,

"house-keeping" genes utilized in miRNA expression normalization are of variable expression across tissues. Even U6 small RNA, often used as the control gene in miRNA qRT-PCR experiments or northern blot, cannot be assumed to be constant. Validation should always be performed to ensure that the control gene is of uniform expression in all experimental conditions if the invariantly expressed gene normalization approach is taken. One way to perform such validation is to evaluate whether the expression of the control gene per cell or per amount of total RNA significantly alters under the experimental setting. Finally, it may not be safe to assume that total miRNA expression is constant across samples [5]. This is because total miRNA levels can be altered in disease conditions [5], upon culture confluency [30], with the activation of the Hippo pathway [31], or subjected to RNA modification events [32].

Given the challenges of normalization, our laboratory has used several empirical rules to determine which method of normalization should be applied as an initial choice. When examining data from a single cell line treated with either genetic agents or chemicals, it is often safe to assume that total miRNA expression remain constant across samples. Exceptions will include conditions that result in differential culture confluency, or the activation of the Hippo pathway, as mentioned above. When it is suspected that global miRNA expression within cells may be altered under the experimental condition or between samples, spike-in of synthetic RNA sequences of miRNA-like chemical structure can be used for normalization [5]. Finally, employing multiple techniques for validating normalization results is always an important consideration.

From Profiling Data to Insights into the miRNA Pathway as Tumor Suppressor in Cancer

miRNA expression profiling has generated specific insights into diverse biological processes, including cancer. To illustrate the importance of data normalization, we will focus on one specific example of how such technical considerations have contributed to the theory that the miRNA pathway functions as a tumor suppressor in cancer.

In examining the expression profiles of solid cancer tissues from multiple cancer types, it has been observed that cancer tissues tend to express globally lower total miRNA levels compared to corresponding normal tissues. This observation was possible due to the use of spike-in synthetic small RNA, and subsequent normalization based on these synthetic RNA signals to reflect miRNA quantities per amount of total RNA. Consistent with this, cancer cell lines express lower amounts of total miRNA as compared to tissue samples [5]. In contrast to these results, a similar study on solid cancer tissues with a normalization method assuming median miRNA expression across samples could not observe this phenomenon [33], suggesting that the choice of normalization method may lead to differences in data interpretation.

The finding of globally lower miRNA expression in cancer can lead to several interesting questions. First, does this lowered global miRNA expression reflect the consequence of reaching a cancerous state, or does lowered miRNA pathway activity play functional roles during tumorigenesis? Second, what are the mechanisms that can result in global miRNA expression changes? Third, other than lowering global miRNA expression, do cancer cells utilize other approaches to avoid miRNA-mediated gene expression control? Seminal studies from multiple laboratories have provided key answers to these questions, which are summarized below.

How can global decrease in miRNA abundance occur in cancer cells? Although miRNAs tend to locate in fragile regions of the genome [34], it is difficult to imagine that genetic loss of specific miRNA loci can contribute to this global decrease. Instead, changes in the miRNA biogenesis pathway are more likely the source. Consistent with this concept, Kumar et al. found that the Dicer1 allele exhibit frequent hemizygous copy number loss in many tumor types [35]. Using a well-defined mutant-K-ras-initiated lung cancer model, Kumar et al. observed that genetic Dicer ablation cooperates with mutant K-ras to initiate tumorigenesis [36]. Interestingly, on the mutant K-ras background, both $Dicer^{fl/+}$ mice and $Dicer1^{fl/fl}$ gave rise to similar levels of increased tumorigenesis [36], and it was later confirmed that the lung tumors formed in both $Dicer1^{fl/+}$ and $Dicer1^{fl/fl}$ mice had only one allele of Dicer1 ablation, with the other allele being wild type, leading to a model that a single-copy loss of Dicer1 promotes tumorigenesis, whereas losing both copies of Dicer1 is incompatible with cancer.

As a modification of this theory, Ravi et al. have found that Dicer null status can be compatible with a cancerous cell state [37]. Using mutant K-ras and p53 null mouse sarcoma as a model, it was shown that Dicer null cells can exist on this compound genetic background, and the resultant Dicer null cells can form tumors in vivo [37]. While Dicer null cancer cells can be propagated as a cell line in vitro, it has also been observed that Dicer null cells show higher levels of apoptosis, suggesting that complete loss of Dicer can result in a tumor with reduced fitness. Similar results have been obtained from a study in which 293T cell lines with complete ablation of Dicer can be propagated in culture. These cells also show decreased fitness in comparison with cells retaining wild-type Dicer alleles [38, 39].

Interestingly, in both studies, a small amount of miRNAs can still be observed in cells with complete loss of Dicer. Even though it is not fully clear whether the remaining levels of miRNAs are functional, these observations raise an interesting question whether complete loss of miRNA expression can be incompatible with a cancerous state. Overall, these studies support that global loss of miRNA expression functionally enhances tumorigenesis. Partial loss of miRNA processing activity enhances tumorigenesis in the presence of other oncogenic mutations, whereas further loss of miRNA processing activity will be detrimental to, although may not completely abrogate, tumorigenic potential.

In addition to Dicer1 copy number alterations, mutations of TRBP2, a Dicer partner protein, have been identified both in human cancer cell lines and in cancer specimens [40]. Recently, high frequency mutations in several miRNA processing pathway genes, including Dicer1, Drosha, DGCR8, and Exportin5, have been

reported in subsets of Wilms tumor [41, 42]. In addition to genetic mutations or copy number alterations, cancer-related signaling pathways can regulate global miRNA processing. For example, Hwang et al. initially found that culturing cells to confluency increases global miRNA expression [30].

Mori et al. further demonstrated that signaling from Hippo pathway, an important cancer pathway, can control the processing of many miRNAs through cis-elements in pri-miRNAs. Specifically, phosphorylated nuclear-localized YAP can sequester an important cofactor in the microprocessor complex, DDX17 (p72), away from pri-miRNAs. In addition to the Hippo pathway, SRSF3 (also known as SRp20) and RNA methylation protein METTL3 have also been linked to pri-miRNA processing control for a large number of miRNA genes [43]. It will be interesting to examine whether such factors can contribute to cancer initiation and/or progression.

Besides alterations in miRNA processing, the fact that cancer cells are more proliferative than normal cells provides a further link to decreased global miRNA expression in cancer. miRNAs are recognized as highly stable in cells [25, 43, 44], and a recent survey across eight mammalian cell types further demonstrated widespread long half-lives of the vast majority of miRNAs [25]. Such high stability means that cells proliferating faster will have increased probability to passively dilute their miRNA pool, and thus leading to further decreased global miRNA levels in cancer cells.

Other than the downregulation of global miRNA levels, cancer cells have a higher tendency to evade miRNA-mediated gene regulation through modulation of alternative polyadenylation events. Sandberg et al. observed that alternative utilization of polyadenylation sites can occur in proliferating cells, which leads to shortened 3′UTRs for many messenger RNAs [45–50]. Mayr et al. specifically examined this effect in cancer, and shortening of 3′UTR in cancer cells leads to evasion of regulation by tumor suppressor miRNAs [51].

Together, the above studies highlight the importance of proper normalization of miRNA expression profiling data because global miRNA expression may be altered in disease or experimental conditions.

Conclusions and Future Prospects

The past decade or so has seen exciting development of methodologies in quantifying miRNA expression profiles, which has not only led to biological insights about miRNA-regulated cellular pathways, but also raised the prospect of miRNA-expression-based diagnostics in clinics. At the same time, insights gained from miRNA profiling will push new technology development and raise fundamental questions about miRNAs. We discuss here the single-cell-level intercellular miRNA expression heterogeneity, and further work to understand the functional impact of intracellular miRNA isoform heterogeneity.

Table 21.1 Comparison of existing single-cell miRNA quantification technologies. Features of single cell miRNA quantification methods, including qRT-PCR, fluorescence in situ hybridization (FISH), and microfluidics RT-PCR are shown

	qRT-PCR	FISH	Microfluidic RT-PCR
Cell number input	A few cells	Many cells	300 Cells
miRNA Multiplexing	>100 miRNAs	~1–3 miRNAs	9 miRNAs
Single cell quantification	Yes	Yes	Yes

The appreciation of cellular heterogeneity in both normal and diseased tissues has led to a demand of single cell technologies to quantify gene expression at the single cell level. For example, it is increasingly recognized that cancer cells are heterogeneous within the same tumor, and in acute myeloid leukemia, rare cancer stem cells are capable of initiating leukemia and evading therapeutic treatment [52–54]. miRNAs profiles, as an important reporter of cellular and diseased states [5], could be very useful in deciphering the heterogeneity within a cell population. Such heterogeneity information might further aid disease diagnosis.

Compared to messenger RNAs, highly expressed miRNAs have the advantage of being more abundant in terms of molecules per cell and with overall longer half-lives, and thus may be less susceptible to noise of transcription at the single cell level. In addition, the ability of measuring miRNA expression in singles cells, coupled with single cell measurements of messenger RNAs, can be a powerful tool to shed light on how miRNAs regulate target genes at the single cell level, which is currently only achievable using reporter systems for a single miRNA on a single target gene [55, 56].

Several methodologies have been demonstrated to detect miRNA expression in single cells (Table 21.1). For example, Tang et al. have demonstrated the feasibility of using multiplex RT and two rounds of amplification to detect expression from ~200 miRNAs in a limited number of single cells [57]. In situ hybridization-based methods are capable of examining a large number of cells, but with one or a few miRNAs [58, 59].

More recently, a microfluidics-based method reported the detection of a few miRNAs in ~300 single cell measurements per microfluidics chip [60]. However, these technologies compromise either on the throughput for the number of miRNAs or on the number of cells to be detected. Ideally, an approach for single cell small RNA sequencing could be possible to provide both cell and miRNA throughput, which also has added benefits of quantifying non-miRNA small RNAs in single cells. This, however, is not currently feasible. To our knowledge, the lowest amount of total RNA reported to generate successful small RNA sequencing [61] is ~5 ng of RNA from serum and body fluids (which may not have the same composition of RNA types as in cells). An additional consideration is that single cells of different sources vary significantly in size, and smaller cells with overall less RNA will demand more sensitive detection. For example, our laboratory's estimate is that mouse hematopoietic progenitors cells have on average ~1–2 pg of total RNA per cell, whereas human embryonic stem cells can have ~10 pg total RNA per cell.

On top of cell size, cancer cells and cancer cell lines tend to have lower amount of miRNAs, which will further add challenges to miRNA detection at the single cell level. Low amount of RNA will likely pose challenges, with low substrate concentration, for biochemical reactions of T4 RNA ligase (Fig. 21.1), which is slow reacting and difficult to push reaction to completion even for larger quantities of RNAs. The advancements in the field of microfluidics may change the landscape of single cell profiling of miRNAs [60]. Microfluidics permit shrinking reaction volumes to the nanoliter range, which substantially increases substrate concentrations that may overcome the limitation of low RNA levels in single cells. With a combination of improvement in biochemical reactions and microfluidics, it might be possible to achieve single cell small RNA sequencing in the next few years.

The appreciation of the coexistence of multiple sequence- or biochemical-isoforms of miRNAs leads to important questions regarding their functions in cells. Can these miRNA isoforms regulate different sets of genes? Do they play distinct biological functions in cells? As an important target-recognition motif for miRNAs, the "seed" region is located at positions 2–7 or 2–8 at the 5′ end of miRNAs [62]. For some miRNAs, it is thus conceivable that isoforms with different "seed" sequences may regulate different sets of target genes. It has been observed in genomic data analyses that a 6-nucleotide region with a single base offset from the traditional seed is also associated with target gene regulation [62]. The regulatory output of such "offset 6mer" cannot be easily explained by crystal structures of miRNA-AGO2 complexes [27, 28]. It is thus possible that the gene regulation effect of the offset-6mer motif is actually mediated by miRNA isoforms in cells. Careful analyses, possibly with synthetic miRNA strands, are necessary to understand the regulatory and functional roles of miRNA isoforms.

References

1. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75(5):843–54; Epub 1993/12/03.
2. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature. 2000;403(6772):901–6; Epub 2000/03/08.
3. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell. 1993;75(5):855–62; Epub 1993/12/03.
4. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 2014;42(Database issue):D68–73; PMCID: 3965103; Epub 2013/11/28
5. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature. 2005;435(7043):834–8; Epub 2005/06/10.
6. Miska EA, Alvarez-Saavedra E, Townsend M, Yoshii A, Sestan N, Rakic P, et al. Microarray analysis of microRNA expression in the developing mammalian brain. Genome Biol. 2004;5(9):R68; PMCID: 522875; Epub 2004/09/04.
7. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell. 2007;129(7):1401–14; PMCID: 2681231; Epub 2007/07/03.

8. Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, et al. MicroRNA expression in zebrafish embryonic development. Science. 2005;309(5732):310–1; Epub 2005/05/28.
9. Duan D, Zheng KX, Shen Y, Cao R, Jiang L, Lu Z, et al. Label-free high-throughput microRNA expression profiling from total RNA. Nucleic Acids Res. 2011;39(22), e154; PMCID: 3239174; Epub 2011/10/07.
10. Chapin SC, Appleyard DC, Pregibon DC, Doyle PS. Rapid microRNA profiling on encoded gel microparticles. Angew Chem Int Ed Engl. 2011;50(10):2289–93; PMCID: 4104285; Epub 2011/02/26.
11. Baker M. MicroRNA profiling: separating signal from noise. Nat Methods. 2010;7(9):687–92; Epub 2010/09/02.
12. Friedlander MR, Chen W, Adamidi C, Maaskola J, Einspanier R, Knespel S, et al. Discovering microRNAs from deep sequencing data using miRDeep. Nat Biotechnol. 2008;26(4):407–15; Epub 2008/04/09.
13. Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science. 2001;294(5543):858–62; Epub 2001/10/27.
14. Pawlicki JM, Steitz JA. Primary microRNA transcript retention at sites of transcription leads to enhanced microRNA production. J Cell Biol. 2008;182(1):61–76; PMCID: 2447899; Epub 2008/07/16.
15. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281–97; Epub 2004/01/28.
16. Starega-Roslan J, Krol J, Koscianska E, Kozlowski P, Szlachcic WJ, Sobczak K, et al. Structural basis of microRNA length variety. Nucleic Acids Res. 2011;39(1):257–68; PMCID: 3017592; Epub 2010/08/27.
17. Han J, Lee Y, Yeom KH, Nam JW, Heo I, Rhee JK, et al. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell. 2006;125(5):887–901; Epub 2006/06/06.
18. Ameres SL, Horwich MD, Hung JH, Xu J, Ghildiyal M, Weng Z, et al. Target RNA-directed trimming and tailing of small silencing RNAs. Science. 2010;328(5985):1534–9; PMCID: 2902985; Epub 2010/06/19.
19. Newman MA, Mani V, Hammond SM. Deep sequencing of microRNA precursors reveals extensive 3′ end modification. RNA. 2011;17(10):1795–803; PMCID: 3185913; Epub 2011/08/19.
20. Burroughs AM, Ando Y, de Hoon MJ, Tomaru Y, Nishibu T, Ukekawa R, et al. A comprehensive survey of 3′ animal miRNA modification events and a possible role for 3′ adenylation in modulating miRNA targeting effectiveness. Genome Res. 2010;20(10):1398–410; PMCID: 2945189; Epub 2010/08/20.
21. Chiang HR, Schoenfeld LW, Ruby JG, Auyeung VC, Spies N, Baek D, et al. Mammalian microRNAs: experimental evaluation of novel and previously annotated genes. Genes Dev. 2010;24(10):992–1009; PMCID: 2867214; Epub 2010/04/24.
22. Fernandez-Valverde SL, Taft RJ, Mattick JS. Dynamic isomiR regulation in Drosophila development. RNA. 2010;16(10):1881–8; PMCID: 2941097; Epub 2010/09/02.
23. D'Andrade PN, Fulmer-Smentek S. Agilent microRNA microarray profiling system. Methods Mol Biol. 2012;822:85–102; Epub 2011/12/07.
24. Wang H, Ach RA, Curry B. Direct and sensitive miRNA profiling from low-input total RNA. RNA. 2007;13(1):151–9; PMCID: 1705746; Epub 2006/11/16.
25. Guo Y, Liu J, Elfenbein SJ, Ma Y, Zhong M, Qiu C, et al. Characterization of the mammalian miRNA turnover landscape. Nucleic Acids Res. 2015;43(4):2326–41; PMCID: 4344502; Epub 2015/02/06.
26. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005;33(20), e179; PMCID: 1292995; Epub 2005/11/30.

27. Schirle NT, MacRae IJ. The crystal structure of human Argonaute2. Science. 2012;336(6084):1037–40; PMCID: 3521581; Epub 2012/04/28.
28. Frank F, Sonenberg N, Nagar B. Structural basis for 5′-nucleotide base-specific recognition of guide RNA by human AGO2. Nature. 2010;465(7299):818–22; Epub 2010/05/28.
29. Lee RC, Ambros V. An extensive class of small RNAs in Caenorhabditis elegans. Science. 2001;294(5543):862–4; Epub 2001/10/27.
30. Hwang HW, Wentzel EA, Mendell JT. Cell-cell contact globally activates microRNA biogenesis. Proc Natl Acad Sci U S A. 2009;106(17):7016–21; PMCID: 2678439; Epub 2009/04/11.
31. Mori M, Triboulet R, Mohseni M, Schlegelmilch K, Shrestha K, Camargo FD, et al. Hippo signaling regulates microprocessor and links cell-density-dependent miRNA biogenesis to cancer. Cell. 2014;156(5):893–906; PMCID: 3982296; Epub 2014/03/04.
32. Yang W, Chendrimada TP, Wang Q, Higuchi M, Seeburg PH, Shiekhattar R, et al. Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nat Struct Mol Biol. 2006;13(1):13–21; PMCID: 2950615; Epub 2005/12/22.
33. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006;103(7):2257–61; PMCID: 1413718; Epub 2006/02/08.
34. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A. 2004;101(9):2999–3004; PMCID: 365734; Epub 2004/02/20.
35. Kumar MS, Pester RE, Chen CY, Lane K, Chin C, Lu J, et al. Dicer1 functions as a haploinsufficient tumor suppressor. Genes Dev. 2009;23(23):2700–4; PMCID: 2788328; Epub 2009/11/12.
36. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet. 2007;39(5):673–7; Epub 2007/04/03.
37. Ravi A, Gurtan AM, Kumar MS, Bhutkar A, Chin C, Lu V, et al. Proliferation and tumorigenesis of a murine sarcoma cell line in the absence of DICER1. Cancer Cell. 2012;21(6):848–55; PMCID: 3385871; Epub 2012/06/16.
38. Bogerd HP, Whisnant AW, Kennedy EM, Flores O, Cullen BR. Derivation and characterization of Dicer- and microRNA-deficient human cells. RNA. 2014;20(6):923–37; PMCID: 4024645; Epub 2014/04/24.
39. Flores O, Kennedy EM, Skalsky RL, Cullen BR. Differential RISC association of endogenous human microRNAs predicts their inhibitory potential. Nucleic Acids Res. 2014;42(7):4629–39; PMCID: 3985621; Epub 2014/01/28.
40. Melo SA, Ropero S, Moutinho C, Aaltonen LA, Yamamoto H, Calin GA, et al. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. Nat Genet. 2009;41(3):365–70; Epub 2009/02/17.
41. Walz AL, Ooms A, Gadd S, Gerhard DS, Smith MA, Guidry Auvil JM, et al. Recurrent DGCR8, DROSHA, and SIX homeodomain mutations in favorable histology Wilms tumors. Cancer Cell. 2015;27(2):286–97; Epub 2015/02/12.
42. Wegert J, Ishaque N, Vardapour R, Georg C, Gu Z, Bieg M, et al. Mutations in the SIX1/2 pathway and the DROSHA/DGCR8 miRNA microprocessor complex underlie high-risk blastemal type Wilms tumors. Cancer Cell. 2015;27(2):298–311; Epub 2015/02/12.
43. Kai ZS, Pasquinelli AE. MicroRNA assassins: factors that regulate the disappearance of miRNAs. Nat Struct Mol Biol. 2010;17(1):5–10; Epub 2010/01/07.
44. Ji L, Chen X. Regulation of small RNA stability: methylation and beyond. Cell Res. 2012;22(4):624–36; PMCID: 3317568; Epub 2012/03/14.
45. Rowley JD. Chromosome translocations: dangerous liaisons revisited. Nat Rev Cancer. 2001;1(3):245–50; Epub 2002/03/21.
46. Sambrook J, Russell DW. Separation of RNA according to size: electrophoresis of glyoxylated RNA through agarose gels. CSH Protoc. 2006; 2006(1); Epub 2006/01/01
47. Sandberg R, Neilson JR, Sarma A, Sharp PA, Burge CB. Proliferating cells express mRNAs with shortened 3′ untranslated regions and fewer microRNA target sites. Science. 2008;320(5883):1643–7; PMCID: 2587246; Epub 2008/06/21.

48. Spies N, Burge CB, Bartel DP. 3′ UTR-isoform choice has limited influence on the stability and translational efficiency of most mRNAs in mouse fibroblasts. Genome Res. 2013;23(12):2078–90; PMCID: 3847777; Epub 2013/09/28.
49. Ross AF, Oleynikov Y, Kislauskis EH, Taneja KL, Singer RH. Characterization of a beta-actin mRNA zipcode-binding protein. Mol Cell Biol. 1997;17(4):2158–65; PMCID: 232064; Epub 1997/04/01.
50. Ruther U, Garber C, Komitowski D, Muller R, Wagner EF. Deregulated c-fos expression interferes with normal bone development in transgenic mice. Nature. 1987;325(6103):412–6; Epub 1987/01/04.
51. Mayr C, Bartel DP. Widespread shortening of 3′UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells. Cell. 2009;138(4):673–84; PMCID: 2819821; Epub 2009/08/26.
52. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001;414(6859):105–11; Epub 2001/11/02.
53. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730–7; Epub 1997/07/01.
54. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 1994;367(6464):645–8; Epub 1994/02/17.
55. Mukherji S, Ebert MS, Zheng GX, Tsang JS, Sharp PA, van Oudenaarden A. MicroRNAs can generate thresholds in target gene expression. Nat Genet. 2011;43(9):854–9; PMCID: 3163764; Epub 2011/08/23.
56. Bosson AD, Zamudio JR, Sharp PA. Endogenous miRNA and target concentrations determine susceptibility to potential ceRNA competition. Mol Cell. 2014;56(3):347–59; Epub 2014/12/03.
57. Tang F, Hajkova P, Barton SC, Lao K, Surani MA. MicroRNA expression profiling of single whole embryonic stem cells. Nucleic Acids Res. 2006;34(2), e9; PMCID: 1351374; Epub 2006/01/26.
58. Lu J, Tsourkas A. Imaging individual microRNAs in single mammalian cells in situ. Nucleic Acids Res. 2009;37(14), e100; PMCID: 2724290; Epub 2009/06/12.
59. Kloosterman WP, Wienholds E, de Bruijn E, Kauppinen S, Plasterk RH. In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. Nat Methods. 2006;3(1):27–9; Epub 2005/12/22.
60. White AK, VanInsberghe M, Petriv OI, Hamidi M, Sikorski D, Marra MA, et al. High-throughput microfluidic single-cell RT-qPCR. Proc Natl Acad Sci U S A. 2011;108(34):13999–4004; PMCID: 3161570; Epub 2011/08/03.
61. Williams Z, Ben-Dov IZ, Elias R, Mihailovic A, Brown M, Rosenwaks Z, et al. Comprehensive profiling of circulating microRNA via small RNA sequencing of cDNA libraries reveals biomarker potential and limitations. Proc Natl Acad Sci U S A. 2013;110(11):4255–60; PMCID: 3600502; Epub 2013/02/27.
62. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2):215–33; PMCID: 3794896; Epub 2009/01/27.

ERRATUM

microRNA: Medical Evidence

Gaetano Santulli

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2

DOI 10.1007/978-3-319-22671-2_22

One of Dr. Santulli's affiliations was incorrect and the other was missing on the copyright page and also the opening page for the below chapter, and have been corrected in the book.

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

The incorrect affiliation reads:

Gaetano Santulli
Columbia University Medical Center
New York, NY, USA

The corrected and missing affiliations are given below:

Gaetano Santulli
Columbia University Medical Center,
New York Presbyterian Hospital —Manhattan, New York, NY, USA

"Federico II" University Hospital, Naples, Italy

The online version of the updated book can be found at
http://dx.doi.org/10.1007/978-3-319-22671-2

G. Santulli, M.D., Ph.D.
Columbia University Medical Center,
New York Presbyterian Hospital —Manhattan, New York, NY, USA

"Federico II" University Hospital, Naples, Italy
e-mail: gsantulli001@gmail.com

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2_22

Index

G. Santulli (ed.), *microRNA: Medical Evidence*, Advances in Experimental Medicine and Biology 888, DOI 10.1007/978-3-319-22671-2